Identification and Visualisation of Actin-Binding Proteins in *Arabidopsis thaliana* and Tobacco BY2 Cells

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Abstract

The cytoskeleton is a remarkable system of filaments that helps in the organisation and functioning of living cells. In plant cells, this cytoskeleton comprises actin microfilaments and microtubules that polymerise from actin and tubulin respectively. While these proteins are highly conserved in eukaryotes, the plant cytoskeleton performs many plant-specific functions. The organisation and functions of the cytoskeleton are determined by a plethora of accessory proteins (actin-binding proteins, microtubule-associated proteins) that link the cytoskeletal filaments to other cell components and to each other. While there is extensive data for the subcellular localisation of actin-binding proteins with actin microfilaments in animal cells, surprisingly few experiments of this type have previously been tried in plants, and the subcellular localisation of most plant actin-binding proteins remains unknown. Such information is important in assessing functions of these proteins to give a better understanding of the actin cytoskeleton. In this study, an attempt was made to visualise the association of actin microfilaments and actin-binding proteins. A range of antibodies raised against various plant and animal actin-binding proteins were screened in two model systems for plant cytoskeleton research, the root of *Arabidopsis thaliana* and in whole cells of the tobacco BY2 liquid cell culture. Further, because previous data in the localisation of the actin-binding protein tropomyosin have suggested that the localisation of this actin-biding protein with the finer cortical actin microfilaments in *Arabidopsis* roots might not be discerned due to high cytoplasmic background, immunolabelling experiments were also conducted on plasma membrane ghosts generated from tobacco BY2 from which any non-specific cytoplasmic labelling could be washed away. There experiments gave some preliminary suggestions for the association of the actin-binding proteins to the actin cytoskeleton in plant cells. The most intriguing observations were obtained with antibodies against the β-subunit of capping protein which colocalised with larger microfilament bundles in tobacco BY2 cells. No colocalisation was observed on membrane ghosts on which these bundles are not well retained. However, the previous experiments in which there were suggestions of tropomyosin-related proteins associated with fine cortical microfilaments in *Arabidopsis* could not be replicated. As no cytoskeletal localisation was observed in either *Arabidopsis* or tobacco with antibodies raised against known actin-bundling proteins from *Arabidopsis* such as villin and fimbrin, it is speculated that the labelling protocols, currently optimised for visualising the actin cytoskeleton, might not to be modified to allow visualisation of actin-binding proteins in plant cells.

Abbreviations

ABP	actin-binding protein
ADF	actin depolymerisation factor
ARP2/3	actin-related protein 2 and 3 protein complex
BSA	bovine serum albumin
BY2	bight yellow 2 (*Nicotiana tabacum* cell culture)
CAP	cyclase associated protein
CPA	capping protein
DAPI	4, 6-diamidino-2-phenylindole
EB1	end-binding protein 1
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GFP-fABD2	GFP fused to the actin-binding domain of Arabidopsis fimbrin
GFP-MBD	GFP fused to the mcirotubule binding domain of mouse MAP4
MAP	microtubule-associated protein
MBS	3-maleimidobenzoic acid N-hydroxysuccinimide ester
Mops	4-Morpholinepropanesulfonic acid
PBS	phosphate buffered saline
Pipes	1,4-Piperazinediethanesulfonic acid
PME	Pipes Magnesium EGTA buffer
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
VLN	villin
TM	tropomyosin

Chapter 1

Introduction

1 The Eukaryotic Cytoskeleton

The cytoskeleton is a remarkable system of filaments that helps in the organisation and functioning of living cells. The cytoskeleton spatially organises the contents of a cell, connects the cell to the external environment in a physical and biological manner, enables cell movement, and helps to maintain cell shape. An array of cytoplasmic proteins and organelles integrate with the cytoskeleton to achieve these functions (Fletcher and Mullins 2010). The formation of the eukaryotic cytoskeleton depends on three families of proteins that polymerise to form the three types of cytoskeletal filaments. These vary in their mechanical, dynamic and biological properties but have a similar fundamental principle in that they are dynamic protein polymers. The cytoskeleton is composed of:

i) 25 nm-wide microtubule that from dimers of α- and β-tubulin, and which function in determining the position of membrane-enclosed organelles, assist in intracellular transport, and form the mitotic spindle that allows for eukaryotic cell division polymerise,

ii) 7 nm-diameter actin microfilaments that are polymerised from the 42 kDa protein actin, and that influence cell shape and cell movement, and,

iii) 10 to 15 nm wide intermediate filaments that function in providing mechanical strength to the cell and that form from a range of proteins including keratins and lamins.

These three types of cytoskeletal filament are highly conserved in most eukaryotic cells and, for example, are all found in animals and fungi. In plant, however, while the microtubule and microfilaments are highly conserved, classic intermediate filaments appear to be lacking. Moreover, the actin and tubulin proteins that polymerise into the cytoskeleton are also highly conserved across all species. Yeast, plant and human tubulins are 75% identical in amino acid sequence and can be functional interchangeable. Similarly, actins from different species have sequence similarity of about 90% although their functional interchangeability may be lower. Although small variations in tubulin and actin contribute to the difference in function and localisation of the filaments in a cell, the differences in overall structures formed by actin microfilaments and microtubules, and the functions that they perform, are determined by a plethora of accessory proteins that link the filaments to other cell components and also to each other. Proteins that specifically bind to actin are called actin-binding proteins (ABPs) whereas those that interact with microtubules are microtubule-associated proteins (MAPs). These proteins show lower levels of conservation, and while many plant MAPs and plant ABPs have been identified, the organisation of these proteins remains poorly understood in comparison to the ABPs and MAPs present in animal cells.

1.1. Actin and Actin Microfilaments

1.1.1. Actin

Actin is a globular 42 kDa protein, sometimes referred to as G-actin that is present in present in plant cells at intracellular concentrations of up to several hundred micromolar (Gibbon et al. 1999; Snowman et al. 2002; Staiger and Blanchoin 2006; Wang et al. 2005). The actin proteins occurs in cells in two different forms, monomeric G-actin and the polymerised, filamentous F-actin or microfilament The ratio of monomeric to filamentous actin varies between cells but is higher in plants in comparison to yeast or mammalian cells. For example, tobacco suspension cells have as little as 1-2% of actin in filamentous form (Staiger and Blanchoin 2006; Wang et al. 2005) while the figure is about 5 – 10% in maize and field poppy (*Papaver rhoeas*) (Gibbon et al. 1999; Snowman et al. 2002; Staiger and Blanchoin 2006).

The G actin monomer has two major domains. These α and β domains have little contact with each other resulting in formation of a cleft between them. The two major domains are further divided into four sub-domains 1 to 4. The larger cleft between sub-domains 2 and 4 forms the site for binding of divalent cations (Mg^{2+}) and nucleotides (ATP or ADP), whereas the smaller cleft between sub-domains 1 and 3 is hydrophobic in nature due to specific amino acids, and forms a major binding site for accessory proteins and actin-binding proteins (Dominguez 2004; 2007; Dominguez and Holmes 2011; dos Remedios et al. 2003).

1.1.2. Actin Microfilament Structure

The polymerised actin microfilament consists of two parallel protofilaments that twist around each other in a right handed helix. The protofilaments are formed from G-actin monomers assemble in a head-to-tail fashion, thus giving the filaments a distinct structural polarity. The plus end (also referred to as the barbed end based on labelling experiments with myosin visualised by electron microscopy) is the preferred site of polymerisation of further actin monomers while the minus end (or pointed end) is the site of preferential depolymerisation. The F-actin microfilament is also in a constant state of association with nucleotides, with monomers predominantly bound to ATP at plus end and to ADP at the dissociating minus end.

Actin microfilaments are in a constant state of polymerisation and depolymerisation. This mechanism of assembly and disassembly is called treadmilling. Assembly of the actin microfilament occurs by addition of ATP-actin monomers at the plus end and disassembly occurs by loss of monomers from the minus end. ATP-actin is converted to ADP-actin by hydrolysis and this ADP-actin undergoes nucleotide exchange to generate ATP-actin that can be used for new rounds of polymerisation.

1.1.3. Actin–binding proteins

While the actin microfilament is a highly conserved structure, the rate at which it polymerises, depolymerises and structures that the filaments form are determined by a plethora of actin-binding proteins (ABPs) (Ayscough 1998; Staiger 2000). Actin-binding proteins are responsible for maintaining the actin subunit pool, rate of filament turnover, controlling subcellular sites for nucleation and organising the microfilaments into higher order structures. Based on their biochemical activities, actin-binding proteins can be grouped into several categories (dos Remedios et al. 2003; Winder and Ayscough 2005), and in non-plant systems, at least 70 classes of actin-binding proteins have been characterised (Blanchoin et al. 2010; Staiger and Blanchoin 2006).

i) Monomer-binding proteins and filament nucleating proteins. Monomer-binding proteins like profilin, ADF/cofilin, cyclase-associated proteins and thymosin maintain the subunit pool for assembly and disassembly of G-actin. New filaments can form de novo, from the side of existing filaments and by severing of existing filaments. Arp2/3 complex nucleates from the side of an existing filament and caps its minus end. This encourages the addition of monomers to the plus end resulting in rapid elongation of the filament. WASP and WAVE/SCAR proteins enhance the function of Arp2/3 complex. A second major class of actin nucleators are formins. Certain isoforms of formin activate de novo actin nucleation and extension of filament from the plus end and some of them responsible for sequestering monomeric actin. Profilin represses spontaneous nucleation of actin and when the plus ends of the filament are uncapped the profilin-actin complex ferries actin to this end (Deeks and Hussey 2009; dos Remedios et al. 2003; Staiger et al. 2010).

ii) Microfilament-depolymerising proteins. Actin depolymerisation factor (ADF)/cofilin stimulates depolymerisation from the minus end by binding to G- and F-actin. Cofilin also binds to the ADP - actin monomers released during filament turnover and inhibits exchange of their bound nucleotide (Nishida 1985).

iii) Microfilament-bundling and -cross-linking proteins. Actin filaments form higher order structures that are essential for both its form and function of cells. Bundling and cross-linking proteins like fimbrin (Thomas et al. 2009), villin (Thomas et al. 2009), filamin (Bartles 2000), fascin (Kureishy et al. 2002) and α-actinin (Sjöblom et al. 2008) contribute to forming the higher order structure of proteins. These structures are responsible for cellular processes like cell division, expansion and intracellular trafficking (Bartles 2000).

iv) Microfilament-capping and -severing proteins. The growth of filament is controlled by various proteins like capping proteins, gelsolin and tensin which cap the plus end of the filament and

block the addition of new monomers. Contrary to plus-end cappers, minus-end cappers like tropomodulin lead to rapid extension of filament by reducing loss of monomers from the minus end. Gelsolin is also a severing protein that prevents assembly of profilin-actin complex and enhances actin depolymerisation.

v) Microfilament-stabilising proteins. Actin stabilisation proteins like tropomyosin, caldesmon (Huber 1997), nebulins (McElhinny et al. 2003), calponin (El-Mezgueldi 1996) and adducin (Matsuoka et al. 2000) bind along the filament length and stabilise the filament against spontaneous depolymerisation.

vi) Proteins that bind filaments to membranes. Membrane binding proteins like spectrin (DR et al. 2006; Dubreuil 1991; Michelot et al. 2005), α-actinin (Arimura et al. 1988)and formins crosslink the plasma membrane to the cytoskeleton.

vii) The motor protein myosin which is an ATPase that generates directed motion along the actin microfilament. The myosin family of proteins can be divided into at least 15 different subfamilies, based on conservation of the motor domain, the site of both microfilament interactions and the ATPase activity. Animal and fungal cells contain a diverse range of different myosins generating many different forms of intracellular and cellular motility, with the best known example being muscle which is composed of alternating bands of thin filaments (bundle actin microfilaments) and thick filaments (myosin II). In plants, myosin-VIII and myosin XI coated onto organelles are responsible for generating organelle and vesicle motility, visible as cytoplasmic streaming, through interactions with actin microfilament tracks (Shimmen et al. 2000).

1.2. Tubulin and Microtubules

1.2.1. Tubulin

Microtubules (MT) are essential components of the eukaryotic cytoplasm that contribute to important cell functions like determining shape of cells, cell transport, motility and cell division. Microtubules are formed from protein subunits of tubulin. Tubulin subunit is itself a heterodimer composed of two closely related globular proteins call α-tubulin and β-tubulin. The α and β– tubulin have a binding site for GTP and the GTP bound to α-tubulin is trapped at the dimer interface and is never hydrolysed or exchange. In contrast, the β-tubulin can convert between GTP and GDP forms and hydrolysis of GTP has a profound effect on microtubule dynamics. Both the α and β subunits of tubulin exist in different isotypic forms and undergo a variety of post-translational modifications. (Amos and Schlieper 2005; Conde and Cáceres 2009)

1.2.2. Microtubule structure

Microtubule is a hollow cylindrical structure composed of alternating α and β tubulin molecules. Tubulin dimers polymerise end to end to form protofilaments and thirteen laterally associated protofilaments form a single microtubule. Two types of protein-protein contacts are created by the assembly of α and β tubulins. Along the longitudinal axis, the top of one β – tubulin molecule forms an interface with bottom of the α-tubulin molecule in the adjacent heterodimer. Perpendicular to these interactions, protofilaments near them form lateral contacts. Longitudinal and lateral contacts are repeated in a regular pattern to form a helical lattice of the microtubule. These contacts make the microtubules stiff and difficult to bend (Downing and Nogales 1998; Nogales 2000)

1.2.3. Microtubule-Associated Proteins

Microtubule associated proteins or 'MAPs' are proteins that interact with the microtubule cytoskeleton and regulate their function and stability. These proteins were isolated from cell extracts, where 80% was tubulin and remaining 20% was a variety of MAPs. The complexity and composition of these proteins entirely depended on the choice of starting tissue and organism. Originally, MAPs were isolated from mammalian neuronal cells and named according to three size classes of polypeptides, MAP1 (> 250 kDa), MAP2 (~ 200 kDa) and tau protein (50 – 70 kDa) (Mandelkow and Mandelkow 1995). Other proteins classified as MAPs include motor proteins, microtubule severing proteins (katanin), proteins affecting dynamics (Op 18/stathmin), and chaperons that are required for proper folding of tubulin into its native form. Another important microtubule associated protein is the γ tubulin which was first isolated from *Aspergillus* (Oakley and Oakley 1989).The disruption of this gene caused a reduction in the number and length of cytoplasmic microtubules and a complete absence of the mitotic apparatus.(Oakley et al. 1990).

Because the vacuoles, organelles and the cytoplasm in a cell interfere with immunofluorescence studies of the cytoskeleton, membrane ghosts from which these components have been washed away provide an ideal system which to study the interaction of actin and actin-binding proteins, and microtubules and microtubule-associated proteins (Sonobe 1996). However, surprisingly few studies have used membrane ghosts to investigate MAPs and ABPs. These include observations that the 200 kDa microtubule stabilisation protein MOR1 is bound to the cortical microtubules on tobacco BY2 ghosts (Hamada et al. 2004) as is the signalling protein PLD (Gardiner et al. 2001; Marc et al. 1996). Several more putative microtubule interacting proteins have also been identified on BY2 ghosts

(Hamada et al. 2009).Furthermore, tobacco BY2 membrane ghosts have also been used for microtubule polymerisation studies, as ghosts from which microtubules have been removed can repolymerise microtubules through γ-tubulin-dependent microtubule nucleation and polymerisation (Murata et al. 2005).

1.3. Conservation of the Cytoskeleton in Plant Cells

1.3.1. Microtubule

As with all eukaryotes, the cytoskeleton plays critical roles in the growth and development of the plant. Many of these roles are, however, distinct from the functions performed by the cytoskeleton in animal and fungal cells. Microtubules, for example, form the mitotic spindle in plant cells as they do in all other eukaryotes. During interphase, however, plant microtubules do not function in vesicle motility. Instead, it is the cortical microtubules that are adjacent to the plasma membrane which order the deposition of cellulose into the plant cell wall (Bringmann et al. 2012; Paredez et al. 2006). Because of the importance role played the direction of cellulose within the cell wall for cell expansion, the cortical microtubules are therefore critical for the way in which plants, and thus plants as a whole, develop (Bringmann et al. 2012; Lloyd and Chan 2008).

Rather than significant differences in microtubule structure being responsible for the different functions of plant microtubules, it is instead the way that the microtubules are organised by microtubule-associated proteins that result in functional differences. So, while the tubulin dimer proteins are highly conserved between plant and non-plant species, the conservation of microtubule-associated proteins is considerably lower. Various plant microtubule-associated proteins have been discovered (Buschmann and Lloyd 2008; Gardiner and Marc 2003; Hamada 2007; Sedbrook 2004) and only some of these are known from non-plant species.

i) Katanain. This is a microtubule-severing protein which is present and partially conserved in all eukaryotes (Bichet et al. 2001; Burk et al. 2001; McClinton et al. 2001)

ii) MOR1. This protein, a member of the XMAP215 family, is a microtubule-stabilising protein present in all eukaryotes (Hamada et al. 2004; Lechner et al. 2012; Twell et al. 2002; Yasuhara et al. 2002).

iii) Map65. This is a family of microtubule-bundling proteins that are specific to plants, and which contain no homologies to microtubule-associated proteins in other eukaryotes (Gaillard et al. 2008; Mao et al. 2005; Van Damme et al. 2004).

iv) EB1 (End-binding protein 1) is a family of proteins that bind to the plus end of microtubules and which is conserved in all eukaryotes (Bisgrove et al. 2008; Chan et al. 2003; Vaughan 2005).

v) Kinesin is one of the two microtubule motor proteins that use energy from ATP to drive directed motion. Kinesins are divided into 14 different subfamilies based on the organisation of the microtubule-binding motor domain (Lawrence et al. 2004). Unlike the other microtubule motor protein dynein, which is absent from higher plants, the kinesin family of proteins has undergone considerable expansion, notably within specific kinesin subfamilies. The *Arabidopsis* genome contains 61 different kinesins with the majority of these being kinesin-7 and -14s, while rice and poplar also contain large numbers of kinesins (Collings 2009; Malcos and Cyr 2009; Richardson et al. 2006). By comparison, humans contain only 40 kinesins and the yeasts less than 10. The reasons behind of this addition of kinesins remains poorly understood (Collings 2009).

vi) CSI1 (cellulose synthase interactin protein 1) is the protein that links the cellulose synthase complex in the plasma membrane to the cortical microtubules (Bringmann et al. 2012; Li et al. 2012). It lacks similarities to any known microtubule binding proteins.

vii) SPIRAL1 and SPIRAL2 are two plant-specific microtubule-associated proteins that were first discovered through the characterisation of mutants in *Arabidopsis* that had growth defects consistent with microtubule disruption (Nakajima et al. 2004; Sedbrook 2004). These proteins are also unrelated to other known microtubule-associated proteins.

The intracellular localisations of several of these MAPs have been investigated by both GFP fusion proteins and immunofluorescence in plant cells. EB1 isoforms localise to the plus ends of microtubules in the phragmoplast, mitotic spindle and interphase cortical array (Chan et al. 2003; Mathur et al. 2003), while SPIRAL1 also localised to the plus ends of microtubules in all division and endomembranes in *Arabidopsis* seedlings (Sedbrook 2004). MAP65 is a family of microtubule-associated protein, with 9 genes in *Arabidopsis thaliana*, *MAP651 – 9*. AtMAP65-1 is expressed ubiquitously in plant organs during cell cycle and binds to microtubules at specific stages of the cell cycle. The binding of the isotypes was different association and dissociation patterns (Smertenko et al. 2006; Smertenko et al. 2004).

1.3.2. Actin microfilaments

The actin cytoskeleton plays important roles in the functioning of plant cells by contributing to its cell division and development (Burgos-Rivera et al. 2008; Deeks et al. 2007), cell patterning and positioning (Kleine-Vehn et al. 2006; Schellmann and Hulskamp 2005), vesicle and organelle movement (Lee et al. 2008; Peremyslov et al. 2010), signalling (Šamaj et al. 2006) and biotic responses (Hardham et al. 2008; Hardham et al. 2007; Opalski et al. 2005; Shimada et al. 2006).

It is apparent, therefore, that the functions of the actin cytoskeleton in plants are partially divergent to those in non-plant species where the actin cytoskeleton is typically involved in cell motility. This difference is roles do not come about because of the actin itself - the actin monomer and actin microfilaments are highly conserved between plant and non-plant species but because of the range of different actin-binding proteins present.

The genomes for *Arabidopsis* and other plants have now been completely sequenced which allows for a comparative identification of many actin-binding proteins. The *Arabidopsis* genome was searched for 67 known animal and fungal actin binding proteins, but only 31 possibly matches were detected - many other actin-binding proteins were either not detected or are absent (Hussey et al. 2002), and subsequent searches have generated similar results (Staiger and Hussey 2004). In this discussion of conservation, it is, however, important to note that 'conservation' does not imply the >90% conservation present in the actin monomer across all eukaryotes. Instead, actin-binding proteins are significantly less well conserved with amino acid similarities in the range of 30 - 50%.

Actin-binding proteins that have been detected in plants, and that are conserved between plants and other organisms, are listed below grouped by their dominant roles as they were in section 1.1.3.

i) Monomer-binding proteins and filament nucleating proteins include profilin which was first recognised in plants as a pollen protein that is a causative agent for allergies (Valenta et al. 1991) and which has been shown to be widespread in plant cells. Plant profilin are divergent from non-plant species and different profilin isoforms within a single plant may only share 75% sequence similarity (Staiger et al. 1997; Vantard and Blanchoin 2002). Plants also contain the ARP2/3 protein complex involved in the nucleation of actin microfilaments (Frank et al. 2004; Harries et al. 2005; Le et al. 2006; Szymanski 2005). Plants, however, lack β-thymosin, an animal actin binding and sequestering protein found in high concentrations in vertebrate cells.

ii) Microfilament-depolymerising proteins including ADF/cofilin are present in plants (Kim et al. 1993; Rozycka et al. 1995) but share as little as 30% sequence similarity with animal homologues (Staiger et al. 1997).

iii) Microfilament-bundling and -cross-linking proteins. The *Arabidopsis* genome contains nine sequences relating to the bundling protein fimbrin, and AtFim1 shares about 40% amino acid identity with fimbrins of previously characterised fimbrins (Kovar et al. 2000b; McCurdy and Kim 1998). Fluorescently-tagged recombinant fimbrin that has been microinjected into cells has been used o label actin microfilaments in plants (Kovar et al. 2001), and the expression of GFP-fABD2 (GFP fused to the actin-binding domain of *Arabidopsis* fimbrin is one of the better characterised live cell markers for plant actin (Maisch et al. 2009; Sheahan et al. 2004; Wang et al. 2004). The bundling protein villin has also been detected in plant cells (Huang et al. 2005; Khurana et al. 2010; Klahre et al. 2000; Yokota et al. 2005; Yokota et al. 2003; Zhang et al. 2010; Zhang et al. 2011). Other cross-linking and bundling proteins like α-actinin, filamin and fascin were absent in the *Arabidopsis* genome (Drobak et al. 2004; Hussey et al. 2002).

iv) Microfilament-capping and -severing proteins. The α- and β- subunits of capping protein have been detected in plants (Hopmann R et al. 1996; Huang et al. 2003; Huang et al. 2006).

v) Microfilament-stabilising proteins. The conservation of tropomyosin between animals and yeast, and the fundamental roles tropomyosin appears to play in both systems, suggests that a tropomyosin homologue might be present in plants (Drøbak et al. 1994). While tropomyosin sequences have not been detected in the genomes of higher plants, proteins immunologically-related to tropomyosin are present and, seemingly, associated with the actin cytoskeleton (Faulkner et al. 2009).

vi) Proteins that bind filaments to membranes have not been detected in plants although α-Actinin is a crosslinking protein belonging to the spectrin superfamily that is conserved across protozoa, green algae, fungi, insects, birds and mammals. However, this class of protein was found to be absent from homology-based searches of the completed *Arabidopsis* genome(Drøbak et al. 2004; Hussey et al. 2002).

vii) The motor protein myosin is present in plants. First detected through molecular assays (Kinkema et al. 1994; Moepps et al. 1993; Plazinski et al. 1997), plant myosins belong to myosin classes VIII and XI, and their intracellular localisation and functions have been widely characterised (Liu et al. 2001; Reichelt et al. 1999; Reisen and Hanson 2007; Shimmen et al. 2000).

1.4. Model Systems for Studying the Plant Cytoskeleton

1.4.1. *Arabidopsis thaliana*

Arabidopsis is a small weedy plant of the mustard family that serves as a model system for understanding fundamental biological questions related to structure and function of eukaryotes. Numerous attributes have lead *Arabidopsis* to become the most commonly used plant for molecular and cell biology. These include the plant's small size (mature plants reach up to 15-20 cm in height), rapid growth cycle (6 to 8 weeks from germination to maturation), easy maintenance (plants are happy growing in the laboratory, on agar plates and under artificial lights) and fecundity (mature plants produce many, self-compatible flowers, and can generate as many as 5000 seeds per plant) (Koornneef and Meinke 2010). The plant also has a small genome size of about 27000 genes organised into five chromosomes, and was the first plant for which the entire genome sequence was available (TAG 2000). The completed *Arabidopsis* genome sequence has provided insights into plant evolution, chromosomal organisation, genetic regulatory mechanisms, plant development and differentiation, metabolism and signalling mechanisms (TAG 2000). Forward and reverse genetic mutational studies in *Arabidopsis* have helped study many cellular and physiological functions associated with seed formation, flowering, leaf and root development, the different signalling pathways, responses to hormones and pathogens. Also, the study of the natural genetic variation in *Arabidopsis* plant can answer questions of population genetics and evolution (Koornneef and Meinke 2010; Meinke et al. 1998; Page and Grossniklaus 2002).

The cytoskeleton has been extensively and intensively studied in numerous different cell types in *Arabidopsis*. For example, the cytoskeletal basis for trichome morphogenesis (Deeks et al. 2004; Oppenheimer et al. 1997; Schellmann and Hulskamp 2005), pollen tube growth(Fu et al. 2005; Fu et al. 2002; Šamaj et al. 2006; Staiger et al. 2010; Staiger et al. 1994) and pavement cell development (Baluška et al. 2001; Fu et al. 2005; Fu et al. 2002) have all been characterised to varying levels of complexity at the molecular level. The *Arabidopsis* root is not exception to this. The organisation of microtubules in the *Arabidopsis* root has been extensively documented using both immunofluorescence microscopy (Baskin TI et al. 1992; Baskin et al. 1994; Sugimoto et al. 2000) and with GFP-fusion proteins (Camilleri et al. 2002; Granger and Cyr 2001; Ueda et al. 1999), and in both wild-type and an extensive range of microtubule-related mutants (Bichet et al. 2001; Ishida et al. 2007; Nakajima et al. 2004; Sedbrook 2004).The organisation of actin microfilaments have also been extensively studied in *Arabidopsis* roots, again by a combination of immunofluorescence microscopy (Collings and Wasteneys 2005; Wasteneys and Collings 2007) and GFP fusion proteins (Voigt et al. 2005). Disruption of the actin microfilament cytoskeleton has also been extensively studied. To undersatand the importance of actin cytoskeleton in cellular processes and plant development, actin

depolymerising drugs are incorporated. Latrunculin and cytochalasins are actin depolymerising drugs that prevent the polymerisation of globular actin to its filamentous form leading to net depolymerisation leading to deterioration of cell growth (Gibbon et al. 1999; Ketelaar 2002; Thomas et al. 2006). Mutations in the *Arabidopsis* actin genes also cause severe morphological changes thus affecting its growth (Gilliland et al. 2003; Kandasamy et al. 2002; McKinney et al. 1995)(Gilliland et al. 2003; Kandasamy et al. 2002; McKinney et al. 1995; Ringli et al. 2002) These experiments provide a good background to investigate the association of actin with its actin-binding proteins in *Arabidopsis* root cells. (Ringli et al. 2002)

1.4.2. Tobacco BY2 Cells

The suspension culture of tobacco BY2 (Bright Yellow-2) cells is an important biological model system due its virtue of a fast growth, easy maintenance and its ability to attain high degree of synchronised cell cycle. Originating in the early 1970s in Japan, this cell line has been used extensively for physiological, biochemical, cell and molecular research (reviewed in (Geelen and Inzé 2001; Nagata 1992; Shibaoka 1993; Shibaoka et al. 1996; Sonobe 1996; 1997). Numerous studies have been conducted to understand the organisation and functioning of the plant cytoskeleton. In investigations of the microtubule cytoskeleton, both immunofluorescence microscopy (Akashi et al. 1988; Hasezawa et al. 1988; Katsuta and Shibaoka 1988); reviewed in (Shibaoka 1993; Shibaoka et al. 1996; Sonobe 1996; 1997) and subsequently the use of GFP-fusion proteins such as GFP-MBD and GFP-α-tubulin6 (Van Damme et al. 2004) have been used to track the microtubule organisation and dynamics through the cell cycle. The organisation and dynamics of the actin microfilaments have also been studied extensively in whole cells, again using a combination of microscopy (Collings et al. 1998; Hasezawa et al. 1994; Hasezawa et al. 1998; Higaki et al. 2006; Ou et al. 2002; Sonobe and Shibaoka 1989; Szechynska-Hebda et al. 2006) and the use of GFP-fusion proteins such as GFP-mTalin (Kost et al. 1998) and GFP-fABD2 (Maisch et al. 2009; Sheahan et al. 2004)

Tobacco BY2 cells have been used extensively to investigate the biochemistry and molecular biology of the cytoskeleton, and for purifications of numerous different microtubule associated proteins and actin-binding proteins. These include kinesin and kinesin-related proteins (Asada et al. 1997; Asada and Shibaoka 1994; Gotoh and Asada 2007; Matsui et al. 2001), MOR1, a microtubule bundling protein from the XMAP215 family (Hamada et al. 2004; Yasuhara et al. 2002), a 90 kDa phospholipase D with microtubule-binding activity (Gardiner et al. 2001; Marc et al. 1996), a 190 kDa plant-specific MAP thought to regulate interactions between microtubules and actin

microfilaments (Igarashi et al. 2000) and a class of plant-specific MAPs (MAP65) (Smertenko et al. 2000).While the organisation of MAPs within BY2 cells has been explored (Gardiner and Marc 2003; Hamada et al. 2004; Marc et al. 1996; Meng et al. 2010; Wicker-Planquart et al. 2004), somewhat surprisingly, the localisation of actin-binding proteins in tobacco BY2 cells remains largely unexplored.

1.4.3. Plasma Membrane Ghosts

The plasma membrane ghost technique was first introduced by Marchant using protoplasts (cell wall-free cells) isolated from the green alga *Mougeotia* (Marchant 1978). In this method, protoplasts attached to a surface are burst, the cytoplasmic contents washed away, and the proteins and structures associated with the plasma membrane preserved for observation. Initial observations by scanning and transmission electron microscopy on these ghosts indicated that microtubules

were attached to the plasma membrane (Marchant 1978). Electron microscopy images from various plants including *Mougeotia* demonstrated that not only were microtubules associated with the plasma membrane (Marchant 1978; van der Valk and Fowke 1981) but so too were clathrin-coated vesicles (Fowke et al. 1983; van der Valk and Fowke 1981; Wiedenhoeft 1985), unidentified thin cytoskeletal filaments suggested to be actin microfilaments (Doohan and Palevitz 1980; Fowke et al. 1983; van der Valk and Fowke 1981) and endoplasmic reticulum (Hepler et al. 1990; Wiedenhoeft 1985).

Immunofluorescence microscopy greatly improved the speed with which ghost samples could be observed, and allowed physiological investigations of microtubules. These studies typically used tobacco BY2 membrane ghosts (Akashi and Shibaoka 1991) although on occasions other cell culture lines have been used including carrot cells (Cyr 1991) and have demonstrated amongst other things that microtubules were susceptible to cold- and calcium-induced depolymerisation (Cyr 1991; Marc et al. 1996; Mizuno 1992), and to disruption when cells were treated externally with proteases (Akashi and Shibaoka 1991) and reviewed in (Sonobe 1996).

Following on from early demonstrations that 7 nm-diameter cytoskeletal filaments could be observed by electron microscopy (Doohan and Palevitz 1980; Fowke et al. 1983; Kobayashi 1996; van der Valk and Fowke 1981) showed that actin microfilaments associated with the plasma membrane of ghosts made from *Zinnia* cells (Kobayashi 1996)(Kobayashi 1996). Subsequently, it was also demonstrated that membrane ghosts made from tobacco BY2 cells retained both actin microfilaments and microtubules, and that these directly interacted with one another (Collings et al. 1998; Collings et al. 1999).

Because the vacuoles, organelles and the cytoplasm in a cell interfere with immunofluorescence studies of the cytoskeleton, membrane ghosts from which these components have been washed away

provide an ideal system which to study the interaction of actin and actin-binding proteins, and microtubules and microtubule-associated proteins (Sonobe 1996). However, surprisingly few studies have used membrane ghosts to investigate MAPs and ABPs.

1.5. Thesis Objectives

The primary objective of the thesis was to investigate the association of the actin-binding proteins with actin microfilaments in plant cells. Surprisingly, this type of experiment has few precedents in plant cells. With the exception of the motor protein myosin, which localises to actin microfilament bundles and to the ends of cells (Baluška et al. 2000; Reichelt et al. 1999) and the actin microfilament bundling protein EF1α that binds to the large microfilament bundles of the green alga *Nitella* (Collings et al. 1994) and a protein or proteins immunologically-related to tropomyosin that binds to *Chara* microfilament bundles and plasmodesmata, and to plasmodesmata and the cell plate in *Arabidopsis* (Faulkner et al. 2009), reports on subcellular localisations of plant actin-binding proteins are lacking. This stands in contrast to animal cells where there is an extensive and varied literature on the localisations inside cells of most ABPs, with this information being important for assessing the functions of these proteins.

Unpublished data from the Faulkner et al. (2009) also study suggested that there may also be labelling with the polyclonal tropomyosin antibodies to the finer cortical actin microfilaments, although this was difficult to discern against a relatively high level of cytoplasmic fluorescence. Because the antibodies used for labelling were polyclonal, and were specific to the sequences for animal tropomyosin, there is a strong possibility of non-specific binding with general cytoplasmic proteins resulting in the relatively high background observed. To eliminate this non-specific cytoplasmic labelling, it was initially planned that immunolabelling experiments with the tropomyosin antibody would be conducted on membrane ghosts generated from tobacco BY2 cells using methods modified from previously published experiments (Collings et al. 1998; Collings et al. 1999). During the course of the experiments, the thesis goals were expanded in two ways.

i) A range of polyclonal antibodies against animal actin-binding proteins were purchased. These antibodies had previously been used to detect bands on western blots of plant extracts, although the proteins that they react to remain unknown.

ii) A collection of polyclonal antibodies against plant actin-binding proteins isolated from *Arabidopsis thaliana* became available from Dr Chris Staiger (Purdue University, West Lafayette, Indiana USA).

These two sets of antibodies, along with the tropomyosin antibodies, were to be tested by immunofluorescence in *Arabidopsis* roots and with membrane ghosts. Western blotting experiments

will be performed for the actin-binding proteins and actin and tubulin antibodies will be used as controls for western blotting and immunolabelling experiments. The backgrounds on the different proteins targeted by these different antibodies are given in the following sections.

1.5.1.Tropomyosin

Tropomyosin is an actin microfilament stabilising protein (Ujfalusi et al. 2012) that is well conserved in animals and yeast. By immunofluorescence tropomyosin localises with microfilaments in both muscle and non-muscle cells(Lehman et al. 2000). In animal cells, four genes TM1, TM2, TM3 and TM4 encoding for 20 different isoforms of tropomyosin ranging between 28kDa and 33kDa have been identified (Gunning et al. 2005; Perry 2001).TM1, TM2 and TM3 are expressed in muscle cells where the protein associates with troponin to regulate the calcium-sensitive interaction of the myosin with actin and TM4 encodes for a non-muscle tropomyosin proteins. Non-muscle and smooth cells lack the troponin complex and the regulation of this gene in interacting with actin and myosin is modulated in a calcium-sensitive manner in the presence of two calmodulin-regulated proteins, myosin light kinase and caldesmon (Gunning et al. 2005; Pittenger et al. 1994). Tropomyosin stabilises the actin microfilaments and greatly reduces the rate of depolymerisation (Broschat et al. 1989; Hitchcock-DeGregori et al. 1988; Weigt et al. 1990). Two genes encoding for tropomyosin have been identified in yeast, TPM1 and TPM2. They share approximately 20% amino acid identity with animal protein and have smaller molecular sizes of 19 and 23.5kDa. TPM2 shares 64.5% sequence identity to TPM1 and they influence each other's actin binding properties but they have very distinct functions in yeast. TPM1 necessitates stabilisation of actin bundles and vesicular transport. TPM2 is important in the formation of contractile ring during cell cycle and was shown to co-localise with the F-actin ring during cytokinesis in *Schizosacchyromyces pombe* (Arai et al. 1998).

To date, there is no conclusive evidence for the presence of tropomyosin in the *Arabidopsis* genome (Drøbak et al. 2004; Hussey et al. 2002). Tropomyosin-like proteins have been identified in western blots of *Arabidopsis* (42.5 kDa) and leek (58.5 kDa and 54 kDa) protein extracts(Faulkner et al. 2009). Furthermore, a 64kDa polypeptide was identified from *Lillium* pollen tube extracts, three polypeptides of about 30-38 kDa were identified in wheat callus cells (Turkina et al. 1995) and *Heracleum-sosnowskyi* (Turkina and Akatova 1994). Immuno-localisation studies on the alga *Chara corallina* showed that anti-tropomyosin localised along the sub cortical actin bundles which generate cytoplasmic streaming and also localised to the plasmodesmata. However, labelling of anti-tropomyosin in root cells of *Arabidopsis* recognised only the developing cell plate of the phragmoplast and disappeared after cytokinesis. This is contradictory to the role of tropomyosin which associates with microfilaments as an actin stabilising protein (Faulkner et al. 2009).

To investigate the characteristics and functions of tropomyosin in higher plants, I will carry out further immunolabelling experiments on *Arabidopsis* root and membrane ghosts from tobacco BY2 cells.

1.5.2.Villin

Villin is a microfilament bundling, severing, nucleating and capping protein found in the microvilli of epithelial cells of mammalian intestines (Bretscher and Weber 1980b; Craig and Powell 1980).Five villin-like genes, AtVLN1, AtVLN2, AtVLN3, AtVLN4 and AtVLN5 encoding for the five villin isoforms have been identified in *Arabidopsis* genome. AtVLN 2 and 3 have higher homology with animal villin than AtVLN 1 and 4. These genes are expressed ubiquitously in *Arabidopsis* and related proteins have been identified in other crop plants like banana, carrot, bean, and pear (Klahre et al. 2000). This indicates that villin-like proteins have a more general role in plants. When a GUS-reporter gene was fused to AtVLN1 and AtVLN2 and expressed in *Arabidopsis*, the expression patterns of the two genes was found to be complementary to each other in the roots. ATVLN1 was expressed mainly in the leaf vascular tissue, root tips and trichomes. AtVLN2 was strongly expressed in vasculature of roots, vascular tissue of the anthers and stipules. AtVLN expression was found to be lower in the meristematic tissue and was higher in the epidermal layer of *Arabidopsis* indicating a role in cell differentiation of elongated cells rather than in cell division (Klahre et al. 2000).

All *Arabidopsis* villin isoforms have actin bundling properties, Ca^{2+} dependent filament severing and plus end capping properties (Huang et al. 2005; Khurana et al. 2010; Zhang et al. 2010; Zhang et al. 2011) with the exception of AtVLN1 which cannot sever or cap but can bundle actin filaments in an Ca^{2+} independent manner (Zhang et al. 2010). The importance of these proteins in growth of *Arabidopsis* was demonstrated by loss of function mutants (Zhang et al. 2010; Zhang et al. 2011). Co-localisation of AtVLN4 with actin filaments in *Arabidopsis* pollen was exhibited by GFP- tagged villin and microfilaments labelled with Alexa-phallodoin. At-VLN-GFP construct was found to be distributed evenly in pollen and Alexa-phallodoin-AtVLN specifically co-localised with thick actin filaments in the apical, sub-apical, shank and basal regions of pollen tube and pollen grain. Loss of function mutants resulted in slow root hair growth, changes in rate and route of cytoplasmic streaming and reduced the axial and apical actin bundles in *Arabidopsis* (Zhang et al. 2011).

Two plant villins, P-135-ABP (Yokota et al. 1998) and P-115-ABP (Nakayasu et al. 1998) have been isolated from lily (*Lilium longiflorum*) by biochemical fractionation. P-135-ABP and P-115-APB are homologues and related to *Arabidopsis* villin (Klahre et al. 2000; Yokota et al. 2003). Both proteins bundle actin microfilaments and localised with actin filaments in lily pollen tubes (Yokota et al. 1998; Yokota and Shimmen 1999).

Visualising the co-localisation of this protein will help in characterising the protein and give further insights into its function in the plant.

1.5.3. Fimbrin

Fimbrin is a 68kDa actin-bundling protein that was also initially found in the intestinal microvilli of animals (Bretscher and Weber 1980a). Fimbrin cross-links actin filaments to form tight lateral bundles in a calcium-dependent manner. Unlike other crosslinking proteins such as α-actinin, dystrophin, spectrin, fimbrin is a monomeric protein possessing two actin-binding domains of approximately 30 kDa each and does not need to dimerise (Dubreuil 1991). Yeast fimbrin is encoded by the SAC6 gene that shares an extensive homology with animal fimbrin. By immunofluorescence, yeast fimbrin co-localises with actin bundles and actin patches in budding yeast and recombinant Sac6p experiments indicate the importance of fimbrin in actin stabilisation (Adams et al. 1991; Brower et al. 1995; Kubler and Riezman 1993) and preventing depolymerisation (Skau and Kovar 2010).

Five fimbrin-like genes FIM1, FIM2, FIM3, FIM4 and FIM5 have been identified in the *Arabidopsis* genome and a 77 kDa fimbrin-like protein LI-FIM 1 was isolated from lily (*Lillium longiflorum*).This lily protein is preferentially expressed in pollen was found was found to stabilise actin fringe by cross-linking actin microfilaments into bundles, an important factor in the proper growth of the tips of lily pollen tubes(Su et al. 2012). AtFIM1 codes for a 76 kDa fimbrin-like polypeptide (AtFim1) and shares 40% sequence identity with non-plant fimbrin, but there is little conservation in the two calmodulin-calcium binding domains in their N-terminal.AtFIM1 is expressed in the roots, leaves, stem, flower and siliques of the *Arabidopsis* plant (McCurdy and Kim 1998). *In vivo* (Kovar et al. 2001) and *in vitro* (Kovar et al. 2000b) analysis of AtFim1 suggests that it is a calcium-independent actin crosslinking protein that bundles actin microfilament. AtFIM5 exhibits actin bundling activity which stabilises actin filaments in pollen tubes and pollen grain and loss-of function of FIM5 negatively affects its growth (Wu et al. 2010). A green fluorescent protein (GFP) was tagged to the second actin-binding domain (fABD2) of *Arabidopsis* fimbrin AtFIM1 and this fusion protein was used to study the expression patterns of the actin cytoskeleton in *Arabidopsis* plants (Sheahan et al. 2004; Wang et al. 2004; Wang et al. 2008), suspension cultures of *Nicotiana tabacum* and a legume, *Medicago truncatulata* (Sheahan et al. 2004). This fusion protein proved to be better that the conventional GFP-mTalin expression systems as it has few adverse effect on plant morphology or development (Sheahan et al. 2004; Wang et al. 2008).

Nevertheless, there have been no demonstrations of where fimbrin proteins are localised within plant cells. As the GFP fusion protein (GFP-fABD2) contains only a sub-domain of fimbrin, it is used as an actin microfilament reporter protein and it is not a faithful reporter for fimbrin itself.

1.5.4. Capping Protein

As its name suggests capping proteins (CP) cap the plus ends of actin microfilaments with high affinity thereby preventing the addition or loss of actin subunits during actin turnover (Wear and Cooper 2004). Capping proteins are also known as CapZ or β-actinin in vertebrate muscle and cap32/34 in *Dictyostelium.*These proteins are highly conserved across non-plant taxa including yeasts, vertebrates, flies and worms(Drøbak et al. 2004) and are formed as heterodimer with an α-subunit (32 - 36 kDa) and a β-subunit (28 -32 kDa). Capping protein is regulated by phosphoionositide lipids that are involved in signalling (Drøbak et al. 2004; Staiger and Blanchoin 2006; Wear and Cooper 2004). The function of capping protein depends on the organism, its stage of growth and the other actin-binding protein present in the cells. CapZ in muscle cells is critical for actin assembly of the sacromere, while null mutants of *Drosophilia* show capping protein is important for its viability. *Saccharomyces cerevisiae*, however, loss of capping proteins is not lethal but negatively affects the growth, cell polarity, F-actin levels and cell wall deposition.

The α and β subunits of capping protein have been cloned from *Arabidopsis* and recombinant proteins expressed in bacteria. AtCP caps the barbed ends of actin preventing addition of profilin-actin to this end and suppressing depolymerisation. It binds non-preferentially to actin in both ATP and ADP forms. It was also found to eliminate the initial lag phase during actin polymerisation and increases rate of polymerisation (Huang et al. 2003). AtCP is also responsible for maintaining a small population of F – actin in cells. Capping protein must be inactivated or sequestered to allow assembly at the barbed ends of actin filaments which is regulated by phospholipids like phosphatidylinositol 4,5 – bisphosphate and phosphadic acid which is unique to AtCP (Huang et al. 2006). There have, however, been no experiments to visualise co-localisation of capping proteins with actin microfilaments in plant cells.

1.5.5. CAP Protein

Adenylyl cyclase-associated proteins (CAPs) were first identified in yeast (Fedor-Chaiken et al. 1990) and subsequently in mammals, fungi and plants (Hubberstey and Mottillo 2002). In *S. cerevisiae* CAP serves as an effector of Ras, a gene involved in signalling to regulate cell growth and was found to localise to cortical actin patches (Freeman N L et al. 1996; Lila and Drubin 1997; Yu et al. 1999). In *D. discoideum* CAP localised near the plasma membrane in resting cells and was remobilised during cell movement (Noegel et al. 1999). In mammalian cells, CAP was found diffusely in the cytoplasm

and concentrated at the cell membrane and lamellipodia of migrating fibroblasts (Freeman and Field 2000; Vojtek and Cooper 1993)CAP has been shown to play an important role in cell elongation and development which was elucidated by deletions of the cap gene leading to increase in actin filaments during eye differentiation causing morphological changes in *Drosophila* (Benlali et al. 2000).

An cyclase-associated protein (AtCAP1) similar to the yeast homolog was isolated from *Arabidopsis thaliana*.(Barrero et al. 2002) Overexpression of AtCAP1 in tobacco BY2 suspension cells resulted in loss of actin and disruption of cell division causing defects in growth of transgenic tobacco plants (Barrero et al. 2003). Homozygous plants for *cap1* alleles showed a reduction in stature and morphological disruption of various cell types. The germination efficiency of pollen grains was severely reduced as was the growth rate of pollen tubes and root hairs (Deeks et al. 2007). CAP1 was also demonstrated to be the first plant protein to increase the rate of nucleotide exchange on actin (Chaudhry et al. 2007).

1.5.6. α-Actinin and Caldesmon

α-Actinin is a crosslinking protein belonging to the spectrin superfamily that is conserved across protozoa, green algae, fungi, insects, birds and mammals (Arimura et al. 1988; Blanchard et al. 1989; Sjöblom et al. 2008). It is a homodimer with molecular subunit weight of 93 – 103 kDa and a number of isoforms isolated from muscle and non-muscle cells had different calcium-sensitive actin binding properties (Blanchard et al. 1989; Sjöblom et al. 2008). However, this class of protein was found to be absent from homology-based searches of the completed *Arabidopsis* genome(Drøbak et al. 2004; Hussey et al. 2002).

Several reports do exist, however, for α-actinin-related proteins in plant cells. Using immunoblots, an 80 kDa α-actinin-like protein was identified in pollen of *Lilium davidii*.This protein was found to co-localise with the Golgi apparatus and actin microfilament bundles in pollen tubes of this plant and they suggest that it functions in Golgi-associated vesicle budding and/or sorting with actin microfilaments (Li and Yen 2001). The protein has to which the α-actinin antibody binds has not been identified, and the localisation of the protein has not been investigated in non-reproductive cells such as the *Arabidopsis* root or tobacco BY2 cells.

Caldesmon was first isolated from chicken gizzard(Novy et al. 1991) and eventually found in smooth muscle and non-muscle tissue of sheep(Marston and Smith 1984). This protein is conserved across various species like yeast (Eppinga et al. 2006), insects, birds and mammals. It is a major regulatory component of the smooth muscle microfilaments, but also non-muscle microfilaments. Caldesmon binds to actin, calmodulin, tropomyosin and myosin and is an important substrate to many protein kinases. It acts as an inhibitor of the activation of myosin by actin-tropomyosin complex in a Ca^{2+}

sensitive manner by interacting with Ca^{2+} binding proteins like calmodulin.. No homologues for this protein were found in the *Arabidopsis* genome, and no far caldesmon-like proteins have been identified by immunolabelling.

Chapter 2

Materials and Methods

2.1 *Arabidopsis* Experiments

2.1.1 *Arabidopsis* Seed Sterilisation and Planting

Wild type *Arabidopsis thaliana* seeds were surface sterilised in an Eppendorf tube for 90 - 120 seconds with 1 ml of the sterilising solution containing 50% (v/v) ethanol and 6% (v/v) hydrogen peroxide and then washed extensively in sterilised distilled water.

Under sterile conditions and using a yellow tip with its apical 2 - 3 mm removed, 20 seeds were planted on a standard 90 mm diameter plastic Petri dishes containing 1.2% (w/v) agar (Bacto-agar, Difco Laboratories, Franklin Lakes, NJ, USA) dissolved in Hoagland's solution modified by addition of 3% (w/v) sucrose. The Hoagland growth media contained 2 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 90 µM iron-EDTA complex, 46 µM H_3BO_3, 9.1 µM $MnC1_2$, 0.77 µM $ZnSO_4$, 0.32 µM $CuSO_4$ and 0.11 µM MoO_3 (Baskin TI et al. 1992).

2.1.2 *Arabidopsis* Growth Conditions

Plates containing the seeds were sealed with parafilm and stored at 4°C for 2 days to synchronise their germination. They were then transferred to a growth chamber that was maintained at 21°C under constant light (100 $\mu E.m^{-2}.s^{-1}$) for 4 - 5 days. Once the seedlings had reached the appropriate length of 15 to 20 mm, usually after 4 to 5 days, they were used in experiments.

2.1.3 Immunolabelling of *Arabidopsis* Roots

This protocol was based on previously published methods of labelling actin microfilaments and microtubules (Collings and Wasteneys 2005; Faulkner et al. 2009). Seedlings that were four or five day old were pre-treated with 400 µM 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) in PME buffer (50 mM Pipes pH 7.2, 2 mM EGTA, 2 mM magnesium sulfate) for 5 - 10 min. MBS is a cross-linking agent that is known to stabilise the actin microfilament by covalently binding adjacent monomers (Sonobe and Shibaoka 1989). The seedlings were then fixed in PME containing 3.7% (v/v) formaldehyde, 1% (v/v) glutaraldehyde, 400 µM MBS and 0.1% (v/v) triton X–100 for 40 min or more, and washed twice in PME buffer containing 0.1% (v/v) triton X-100 for 15 min each. They were then extracted with a higher triton X–100 solution (1% (v/v) in PME) for 60 min, and permeabilised with methanol (-20°C, 20 min) followed by rehydration with phosphate buffered saline (PBS) (131 mM sodium chloride, 5.1 mM disodium hydrogen phosphate, 1.56 mM potassium dihydrogen phosphate) for 10 min. A 1 mg/ml sodium borohydride solution was prepared in PBS and seedlings were treated for 20 min. Sodium borohydride reduces aldehydes and ketones to alcohols, and is used to reduce remaining free aldehyde groups present after glutaraldehyde fixations as these are autofluorescent. After extensive PBS washes (a minimum of 3 x 10 min), the cell walls of the

Arabidopsis roots were digested for 10 min with cell wall digest solution that contained 0.1% (v/v) triton X100, 1% (w/v) bovine serum albumin (BSA), 1.0% (w/v) cellulose onozuka R10 (Yakult Pharmaceuticals, Tokyo, Japan), 0.1% (w/v) pectolyase Y23 (MP BioMedicals, Solon OH, USA) and 0.4 M mannitol dissolved in PME buffer. Subsequently, multiple washes with PBS buffer were conducted to completely remove the cell wall digest solution.

To provide maximum mechanical support, and to reduce the possibility of root tips breaking off from the roots, seedlings were carefully laid on parafilm pieces in 30 mm diameter plastic Petri dishes. The roots were laid such that they were overlapping and parallel to each other in a drop of incubation buffer (PBS containing 1% (w/v) BSA and 0.1% (v/v) Tween 20) for 15-20 min. Seedlings were incubated with primary antibodies (see below, Table 2.1) diluted in incubation buffer for 2 hours at room temperature or left overnight at 4°C. They were then washed 3 times for 20 min with PBS. Secondary antibodies of appropriate concentrations (see below, Table 2.2) were also diluted in incubation buffer and incubated for 2 hours at room temperature or overnight at 4°C. After this, seedlings were washed 3 times over twenty minutes with PBS. The nuclei were then labelled with the DNA stain 4, 6-diamidino-2-phenylindole (DAPI, 1 µg/ml in PBS, 10 min). The seedlings were then gently picked out of the dish by their cotyledons and placed on slides and mounted in AF1 antifade agent (Citifluor, London, England). Four drops of nail polish were added to the slide to act as a spacer for the samples, and nail polish was used to seal the coverslip to the slides.

Various concentrations of primary antibodies were used to label the cytoskeleton in these experiments (Table 2.1). Initial experiments focused on methodology development and used mouse monoclonal anti-tubulin diluted 1/1000 and rabbit polyclonal anti-actin diluted 1/400. To characterise actin-binding proteins, samples were double labelled with mouse monoclonal C4 actin and rabbit polyclonal antibodies against the actin-binding protein. Since the working concentrations of these antibodies were unknown, dilution series were carried out for each of the specified actin-binding proteins. C4 actin was used at a known concentration of 1 in 200; the unknown proteins were diluted to 1 in 100, 1 in 400 and 1 in 1000. A list of the primary antibody, concentrations used and the product origin is listed in Table 2. 1.

The secondary antibodies used in these experiments were conjugated with either Alexa fluor dyes, fluorescein or Texas red. These fluorophores absorb light a specific wavelength and emit light at a longer wavelength. Initial labelling experiments used secondary antibodies conjugated to Alexa 546 and fluorescein. As the emission spectra for these dyes slightly overlap, there was the possibility of 'cross-talk' or 'bleed through' between the antibodies, where one antibody is detected in the emission window for the other antibody, thus causing the possibility of false positives. To avoid cross-talk, secondary antibodies conjugated to Texas red and Alexa 594 (which emit further into the red part of

the spectrum) were used instead of Alexa 546,. List of secondary antibody conjugates and the concentrations used are listed in Table 2. 2. Control experiments, in which either one or both of the primary antibodies was omitted from the labelling experiment, but both secondaries were included, were run to verify the specificity of labelling experiments and to eliminate the possibility of cross-talk.

2.2 Tobacco BY2 cells

2.2.1 Growth

Murashige and Skoog medium (MS media) was prepared with pre-packaged salts (4.41 g/L Murashige and Skoog medium, MP Biomedicals, catalogue number 2610020) supplemented by 3% (w/v) sucrose, 2.96 mM thiamine HCl, 0.9 µM 2,4-dichlorophenoxyacetic acid (2, 4-D), 0.55 mM myo-inositol and 1.47 mM potassium dihydrogen phosphate. The pH of media was adjusted to 5.7 with potassium hydroxide, and 100 ml portions were autoclaved in 250 ml conical flasks covered with aluminium foil. Cells were sub-cultured every week with the addition of 2 ml of old culture to the new flask, and were grown on a shaker at 130 rpm and 26°C in constant darkness. 5 ml tips with their end cut off were individually wrapped in aluminium foil and autoclaved. These were used instead of plastic or glass pipettes due to ease of use and to reduce contamination during transfer.

2.2.2 Whole Cell Labelling of Tobacco BY2 Cells

Immunolabelling of whole tobacco BY2 cells followed Collings et al. (1998, 1999), and was conducted with a method similar to that was for *Arabidopsis* root cells with several modifications (Collings et al. 1998; Collings et al. 1999). 20 ml of cell culture was centrifuged (type Z326k centrifuge, Hermle Labortechnik, Wehingen, Germany) at 10,000 rpm for 5 mins and the supernatant discarded. Multiwell slides (MP Biomedicals, catalogue number 6110009) were used for immunolabelling experiments. The wells were coated with 0.1 % (v/v) polylysine for 5 mins, before a rolled tissue was used to wick away the solution. The slides were left to air dry for 5 mins during which time they were covered to prevent dust particles from settling. 20 µl of cells was added to the first 6 wells in a 10 well multiwell slide and left to adhere to the slides for 5 mins. Excess cells and media were then wicked away with tissue paper. The cells were then fixed and processed as with *Arabidopsis* roots, although unlike root cells, BY2 cells were not pre-treated with MBS.

2.2.3 Making Protoplasts

BY2 cells (50 ml) were centrifuged at 1000 rpm for 2 min at 26°C to remove the culture media. Pelleted cells (0.725 ml) were digested in BY2 cell-wall digest (2.0% (w/v) cellulose onozuka R10

(Yakult Pharmaceuticals), 0.1% (w/v) pectolyase Y23 (MP Biomedicals), 2% (w/v) BSA and 0.3 M mannitol to act as an osmoticum. Digests were run in an open scintillation vial at 120 rpm and 26^{o}C for 90 - 120 min. To confirm the production of protoplasts, a drop of digesting cells was placed on a glass slide and checked with a light microscope. Protoplasts appeared round; if the cells were not round then they required further digestion. Protoplasts were centrifuged at 1000 rpm for 3 min and the pelleted protoplasts washed with wash buffer (10 mM Pipes, pH 6.8, 100 mM KCl and 285 mM mannitol) at 1000 rpm for 2 min. The wash buffer was discarded and the protoplasts were re-suspended in 1 ml wash buffer.

2.2.4 Making Membrane Ghosts

This protocol was based on the published methods of Collings et al. (1998, 1999) with several modifications. Multiwell slides containing 10 wells with a diameter of 8 mm were used to prepare and label the samples. Of the 10 wells in the multiwell slide, the first 6 slides were coated with 20 µl of 1 % (v/v) polylysine for 5 min and kept coved to prevent dust fall during coating. Excess polylysine was wiped with tissue paper and the slides were air dried. Protoplast cell suspension (15 µl) was coated on each well for 5 min using a yellow pipette tip with its tip trimmed by 2-3 mm. Wash buffer was wicked away and immediately 20 µl of lysis buffer was added to each well: the cells were lysed my quickly flicking the slide. A further 20 µl of lysis buffer was added and the slide was flicked again. Any remaining cells were burst with a jet of lysis buffer. It is important that the cells were lysed immediately upon removal of wash buffer, as delay between the two steps caused the slide to dry and prevented cell lysis. Cells were fixed for 30 - 60 min in PME buffer containing 3.7% (v/v) formaldehyde, 1% (v/v) glutaraldehyde, 0.1% (v/v) triton X–100 and 1% (v/v) dimethyl sulfoxide (DMSO) with the slides kept covered to prevent evaporation. MBS was not used for pre-treatment of ghosts nor was it used during fixation, as it is not required for actin preservation on ghosts (Collings et al. 1998). Immunolabelling experiments on ghosts were carried out with PME instead of the actin stabilisation buffer (ASB) described previously (Collings et al. 1998; Collings et al. 1999) as it gave sufficiently good preservation of the actin microfilaments. This also allowed direct comparisons with immunolabelling in *Arabidopsis* root cells and tobacco BY2 which were also conducted with PME buffer. Slides were washed with PBS buffer three times over 20 min. Further stabilisation was achieved by air drying slides for 10 min. Cells were then rehydrated with PBS for 5 min, and washed in cold (-20^{o}C) methanol (5 min). After rehydration with PBS (5 min), 0.1 % (w/v) sodium borohydride (20 min) was used to reduce free aldehyde groups followed by further washing in PBS. The cells were blocked with incubation buffer for 10 min and treated with the required concentrations of primary antibody diluted in incubation buffer (30 min) (Table 2.1). The slides were then washed (3 x 10 min in PBS) and treated with secondary antibodies diluted in incubation buffer (30 min) (Table 2.2). The slides were again washed with PBS (3 x 10 min) and the seedlings were treated with

1 µg/ml (DAPI). Slides were wiped with tissue and anti-fade agent Citifluor (Citifluor ltd, London, UK) was added and the slides sealed with nail polish.

2.3. Confocal Microscopy

The *Arabidopsis* root tips, tobacco cells and ghosts were visualised using a confocal microscope (model SP5, Leica, Wetzlar, Germany). 20X NA 0.7 and 63X NA 1.2 glycerol immersion lenses were used to image *Arabidopsis* root cells and tobacco whole cells while a 40X NA 1.3 oil immersion lens was used to image membrane ghosts. Line averaging was set to 5 for whole cells and 8 for membrane ghosts, and images were recorded with sufficient pixels to generate images with resolutions below 100 nm / pixel. Z-stack images with a step size of 1.0 µm were collected for *Arabidopsis* root tips and tobacco BY2 cells. For imaging second antibodies, FITC labelled samples were excited by the 488 nm blue laser and emission was recorded between 496 nm – 530 nm. Samples labelled with Alexa 546, Alexa 594 and Texas red were excited by the 561 nm green laser. Alexa 546 fluorescence was recorded between 570 nm – 617 nm, while fluorescence for Alexa 594 and Texas red were recorded between 590 nm – 620 nm. To avoid crosstalk between the different fluorescent dyes such as FITC and Alexa 546, images were collected using the sequential scan mode so that each dye was imaged separately. Images were processed using LAF AF version 2.6.0 build 7266, Image J (NIH, Bethesda, MD, USA), and Adobe Photoshop CS6.

2.4 Biochemical Techniques

2.4.1 Protein Extraction

Wild type *Arabidopsis thaliana* seeds were planted on agar plates and their roots were harvested after 2 – 3 weeks of growth. 60 seeds were planted per standard plate and a total of 15 plates yielded about 1 g of root material. In order to obtain material from only the roots, the seedlings were picked up by their cotyledons and placed on a clean glass slide. Using a sharp blade, the roots were cut just below the cotyledons and the cotyledons removed. The remaining tissue was then weighed and immediately frozen in liquid nitrogen, and stored at -80°C. BY2 cells were cultured as described in section 2.2.1. A flask containing a 2 – 4 day old BY2 culture yielded about 25 g of cells which were frozen in liquid nitrogen and stored at -80°C.

For protein purifications, 1 g of plant tissue was ground with liquid nitrogen and extracted with 2 ml of RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 0.1% (w/v) sodium dodecyl sulfate, 1 protease inhibitor tablet, 1 mM Na_3VO_4 and 10% (v/v) glycerol) by shaking on ice at 90 rpm for 30 mins. The samples were then centrifuged at 12,000 rpm at 4°C for 10 mins. The pellet was discarded and the supernatant was stored as 100 - 200 µl aliquots at -20°C in

Eppendorf tubes. The concentration of the protein samples were measures using a nanodrop (NanoDrop 1000 spectrophotometer, Thermo Scientific) at 280nm with RIPA buffer as the blank.

2.4.2 Gel Electrophoresis

NuPAGE bis-Tris mini gels (Novex, Life Technologies, Grand Island, NY, USA) were used for gel electrophoresis. Frozen samples were thawed to room temperature and 1X NuPAGE MES running buffer was prepared from a stock solution of 20X (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3) and the gels were run for 35 min at a constant 165 V. The cassette was cracked open and the gel was stained with coomassie brilliant blue stain (0.1% (w/v), methanol (50% [v/v]), glacial acetic acid (10% [v/v]) and visualised using a geldoc (ChemiGenius2 bio imaging system, Syngene, Cambridge, UK).

2.4.3 Western Blotting

Western transfers were used the XCell II^{TM} system (cat no. El9051, invitrogen) were setup following the manufacturer's instructions. Prior to transfer, the nitrocellulose membrane, blotting pads and filter paper were soaked in transfer buffer (50 mM MOPS, 50 mM Tris (pH 7.8), 0.1% (w/v) SDS, 1 mM EDTA). Gels were wet with the transfer buffer and placed on top of the pre-soaked filter paper. Nitrocellulose membrane was then placed on top of the gel and air bubbles were removed by rolling a glass pipette over the membrane surface. Another filter paper was placed on top of the nitrocellulose membrane and air bubbles were again removed with the glass pipette. Pre-soaked blotting pads were placed in the cathode core and the gel-nitrocellulose membrane set-up was carefully placed on top of the blotting pads in the cathode core. The setup was completed by adding more blotting pads on top and placing the anode core such that they form a snug fit. The blot module was then inserted into the lower buffer chamber, and the chamber filled with enough transfer buffers to cover the gel/membrane sandwich. The outer buffer chamber was filled with approximately 600 ml of deionised water which ensured dissipation of heat. The transfer was carried out at a constant voltage of 26 V for 1 hour.

2.4.4 Immunodetection

After the transfer, the nitrocellulose membrane was carefully removed and washed briefly in 10 ml of deionised water. The membrane was then stained with Ponceau red (0.1% (w/v) Ponceau S in 5% acetic acid) to confirm proper transfer of the samples. Immunodetection of proteins was carried out using the WesternBreeze Chromogenic western blot immunodetection kit (Life Technologies). The membrane was blocked in blocking buffer (buffered saline solution containing detergent combined with Hammersten casein soulution at a ratio of 1:2) for 4 h and the primary antibodies were incubated at 4°C overnight on a shaker rotating at 90 rpm. Monoclonal anti-tubulin (clone B512) was diluted 1 in 1000 in blocking buffer while the other primary antibodies were diluted to 1 in 200. After

incubation, membranes were washed in wash buffer (buffer saline solution) (3 x 5 min). Sheep anti-mouse secondary antibodies conjugated to alkaline phosphatase (western breeze kit) were diluted (1 in 5000) with 1X blocking buffer were used to detect mouse monoclonal primary anybodies while sheep anti-rabbit antibodies conjugated to alkaline phosphatase(Sigma Aldrich) (diluted 1 in 5000) were used to detect rabbit polyclonal primaries. The secondary antibodies were incubated at room temperature for 90 min. The membrane was washed with wash buffer three times over 15 mins, and then washed in deionised water to remove wash buffer. The membranes were developed using the Chromogenic substrate for alkaline phosphatase (5-bromo-4 chloro-3 indolyl-1-phosphate (BCIP) and nitro blue tetrazolium (NBT)) in the kit which identified proteins as purple bands on the membrane. The membranes were air dried and scanned (Epson perfection V700 scanner, Epson, Auckland).

Table 2.1. Primary antibodies used in immunoflorescence labelling of *Arabidopsis* roots, and tobacco BY2 cells and ghosts.

Antigen	Antigen source	Concentration	Animal	Source
actin	chicken gizzard smooth actin	1/200	mouse	MP BioMedicals (Solon, OH, USA; clone C4) (Lessard 1989)
actin	maize pollen actin	1/200	rabbit	Dr Chris Staiger (Purdue University, West Lafayette, IN USA) (Gibbon et al. 1999; Ren et al. 1997)
α-tubulin	sea urchin sperm microtubules	1/1000	mouse	Sigma (St Loius, MO, USA; clone B512)
tropomyosin	recombinant chicken smooth muscle tropomyosin	1/100[*1]	rabbit	Abcam (Cambridge, England; antibody 11190) (Faulkner et al. 2009)
fimbrin	*Arabidopsis* recombinant protein	1/100	rabbit	Dr Chris Staiger (Kovar et al. 2000a)
villin	*Arabidopsis* recombinant protein	1/100	rabbit	Dr Chris Staiger (Huang et al. 2005; Khurana et al. 2010; Zhang et al. 2010; Zhang et al. 2011)
caldesmon	residues 750 to the C terminus of human caldesmon	1/100	rabbit	Abcam (Cambridge, England; catalogue number ab68878)
α-actinin	chicken gizzard α-actinin	1/100	rabbit	Sigma (St Loius, MO, USA; catalogue number A2543)
animal villin	residues 7 to 256 of human villin	1/100	rabbit	Abcam (Cambridge, England; catalogue number ab97512)
capping protein α-subunit (CPA)	*Arabidopsis* recombinant protein	1/100	rabbit	Dr Chris Staiger
capping protein β-subunit (CPB)	*Arabidopsis* recombinant protein	1/100	rabbit	Dr Chris Staiger
CAP-1	*Arabidopsis* recombinant protein	1/100	rabbit	Dr Chris Staiger (Chaudhry et al. 2007)

[*1] 1/100 indicates the lowest dilution tested. Experiments also conducted at 1/400 and 1/1000.

Table 2.2. Secondary antibodies used in immunofluorescence

Antigen	Animal	Source	Fluorophore	Code	Conc.	Excitation peak (nm)	Emission peak (nm)	Excitation wavelength (nm) [*1]	Emission wavelengths (nm) [*1]
mouse	sheep	Silenus-Amrad, Boronia, VIC, Australia)	FITC	SAM-F	1/100	494	521	488	496 - 530
	goat	Molecular Probes (Eugene, OR, USA; cat. no. A11003)	Alexa 546	GAM-A546	1/200	556	573	561	570 - 617
	goat	Jackson (West Grove, PA, USA; cat. no. 115-585-003)	Alexa 594	GAM-A594	1/200 / 1/400	591	614	561	590 - 620
	goat	Jackson (West Grove, PA, USA; cat. no. 115-076-003)	Texas red	GAM-TR	1/200	589	615	561	590 - 620
rabbit	sheep	Silenus-Amrad, Boronia, VIC, Australia)	FITC	SARb-F	1/100	494	521	488	496- 530
	goat	Molecular Probes (Eugene, OR, USA; cat. no. A11010)	Alexa 546	GARb-A546	1/200	556	573	561	570 - 617
	goat	Jackson (West Grove, PA, USA; cat. no. 111-585-003)	Alexa 594	GARb-A594	1/200 / 1/400	591	614	561	590 - 620

[*1] Used with confocal microscopy.

Chapter 3

Results

3.1. SDS-PAGE Analysis of Extracted Proteins

Well resolved bands were seen on the SDS-PAGE gels run with crude extracts from both *Arabidopsis* roots and tobacco BY2 cells (Figure 3.1). The extraction procedure was performed using RIPA buffer which proved to be a better system than the other buffers used in which the proteins did not resolve well in the gels and appeared as smeary bands.

3.2. Microfilaments and Microtubules.

Actin microfilaments and microtubules were double labelled using polyclonal anti-actin and monoclonal anti-tubulin (clone B512) and imaged by confocal microscopy in all three experimental systems. Confocal images of roots and whole BY2 cells were collected as optical stacks at 1 μm intervals, and are presented as maximum projections of four or so image planes while images of the optically flat membrane ghosts are single confocal optical sections

Immuno-double labelling of the cytoskeleton in elongating cells of the *Arabidopsis* root demonstrated that actin microfilaments were present in two different arrays. , These were the thick, heavily bundled actin microfilaments that ran predominantly longitudinally within the cell and which drive cytoplasmic streaming, and a finer arrays of cortical microfilaments that were transversely aligned (Figure 3.2A, arrow). The microtubules were exclusively cortical and were aligned transverse to the cell axis (Figure 3.2B). In overlay images (Figure 3.2C), the bundled actin microfilaments (shown in green for this and in all subsequent overlay images, irrespective of the colour of the second antibody used) running longitudinally through the cell stand in stark contrast to the transverse microtubules.

Control experiments demonstrated that there was no cross-talk between the different fluorescence emission channels. If cells were labelled with only the polyclonal anti-actin antibody, but with both secondary antibodies, and imaged under similar settings to Figure 3.2, only actin microfilament labelling was present, and no fluorescence was visible in the microtubule channel (Figure 3.3). Similarly, if only monoclonal anti-tubulin was used in the labelling experiment even in the presence of both secondaries, then microtubules were visible but not fluorescence was detected in the other channel (Figure 3.4). This demonstrated that the secondary antibodies were specific to the primary antibodies used and reduced the likelihood of false positive results seen in subsequent labelling experiments.

In whole tobacco BY2 cells, similar observations were made with control samples. Labelling of transverse microtubule with anti-tubulin (Figure 3.5) and longitudinal microfilament bundles with polyclonal anti-actin (Figure 3.6) showed fluorescence in the appropriate collection window only.

Double labelling of microfilaments and microtubules (Figures 3.7, 3.8) on ghosts made from tobacco BY2 cells showed a random to partial ordered cytoskeleton with varying degrees of co-localisation between the microfilaments and microtubules. In Figure 3.7, there is extensive co-alignment of the actin microfilaments, present predominantly as thinner bundles but with some thicker structures (Figure 3.7A) with the microtubules (Figure 3.7B). However, the overlay image demonstrated that the co-alignment was not perfect, and that in many locations there were unaligned microtubules and microfilaments (Figure 3.7B). The organisation of the actin microfilaments on ghosts was also different to whole tobacco BY2 cells (Figure 3.6) and *Arabidopsis* root cells (Figure 3.2), as in the whole cells, the thick bundles of actin microfilaments were considerably more prominent than on the ghosts. The majority of finer filaments of actin remained separate from the microtubules and co-localisation with microtubules was observed mostly with the thicker actin microfilaments. Three further ghosts showing similar microtubule and microfilament patterns are also shown in Figure 3.8, and labelling with a monoclonal anti-actin antibody (clone C4) also showed similar overall patterns, again with some variability in the amount of bundled actin retained on ghosts (Figure 3.9).

Little could be discerned from the transmitted light images which showed only remnants of the plasma membrane (Figure 3.7D), and transmitted light images were not included for the subsequent images for this reason. Control experiments conducted under similar imaging conditions were conducted on ghosts and showed only actin microfilaments in the presence of the polyclonal anti-actin antibody (Figure 3.10) and microtubules in the presence of the anti-tubulin antibody (Figure 3.11). As there was minimal background in the alternate fluorescence channel, cross-talk between the fluorophores attached to the second antibody had been minimised.

Western blotting experiments demonstrated that the monoclonal anti-tubulin antibody recognised a single band at 55 kDa (Figure 3.12) in both *Arabidopsis* roots and tobacco BY2 cell extracts, which is consistent with the molecular weight of α-tubulin. In contrast, the monoclonal C4 anti actin and polyclonal anti actin did not detect the presence of the 42 kDa actin monomer in either of the samples. Instead, multiple bands at 64, 56 and 47 kDa were detected in tobacco cell samples with the polyclonal anti-actin and a single band at 64 kDa in the root samples was identified (. There were no visible bands on the nitrocellulose membrane for actin using monoclonal C4 actin in either the root samples or the tobacco cell samples, .although this antibody is known to work with western blots (D. Collings, personal communication). The controls shown in the figure are for both mouse and rabbit secondaries. There were no bands visible on the controls which show that the secondary antibodies were specific to the primary antibodies.

3.3. Actin Filament Stabilising Proteins

Labelling in *Arabidopsis* roots with a polyclonal antibody against tropomyosin from chicken smooth muscle showed no distinctive labelling patterns in the distal elongation zone of the root (Figure 3.13). The preliminary observations that were not published in Faulkner et al. (2009) were, therefore, not confirmed. However, a strong signal for tropomyosin was detected in the dividing cells of *Arabidopsis* root cells (Figure 3.14). Dividing cells labelled strongly with the actin antibody (Figure 3.14B, asterisks), an effect previously noted in *Arabidopsis* roots with both antibodies (Collings and Wasteneys 2005; Faulkner et al. 2009) and with GFP fusion proteins (Voigt et al. 2005). These cells also labelled with the tropomyosin antibodies (Figure 3.14A, asterisks), and effect not previously documented by Faulkner et al. (2009). The tropomyosin antibodies also labelled newly formed cell walls and developing cell plates (Figure 3.14A, arrows). In a cell in its latter stages of division (d), tropomyosin was localised mainly to the cell wall and edges of cytoplasm.

Several measures were taken to ensure that the tropomyosin signal (and subsequently those from other actin-binding proteins) was not due to cross–talk from the microfilament labelling. This is first demonstrated by the boxed region (Figure 3.14B,C, boxed region) in which the tropomyosin patterning is clearly different from the actin microfilament labelling shown in panel A - the microfilament signal is not ‘contaminating’ the tropomyosin signal. Second, as most cross-talk between fluorescently-tagged secondary antibodies is from green to the red, the actin-binding proteins such as tropomyosin were labelled with FITC-conjugated secondary antibodies, and the actin microfilaments with a range of red to far-red secondaries. (For consistency, however, actin microfilaments are always shown in green in the colour overlay images.) And third, further control experiments were conducted. Inclusion of only the monoclonal anti-actin antibody, with detection by both secondaries and using standard imaging conditions, showed only the actin microfilaments. No labelling was visible in the ‘ABP channel’ (Figure 3.15B).This control sample also acts as the control for subsequent actin-binding protein localisations in *Arabidopsis* roots.

In contrast to labelling patterns seen in *Arabidopsis*, labelling for tropomyosin in tobacco BY2 cells showed no evidence of preferential co-localisation associated with dividing cells or newly formed cells (Figure 3.16). It was tempting to speculate that lines were present within some images of tropomyosin labelled ghosts (Figure 3.17C, F arrows), but no widespread co-localisation with microfilaments was observed. Controls in which the polyclonal anti-tropomyosin were left out of labelling of whole BY2 cells (Figure 3.18) and ghosts (Figure 3.19) were completely negative in the ‘ABP’ channel, and these act as controls for all of the polyclonal anti-actin binding protein antibodies tested.

Tropomyosin was recognised as a single at 71 kDa in the tobacco cell samples and a specific band at 60 kDa were seen in the *Arabidopsis* root samples (Figure. 3.20). These bands are considerably different from those previously reported, which were 42.5kDa in *Arabidopsis* and 58.5 kDa and 54 kDa in leek (Faulkner et al. 2009).Anti-caldesmon labelling was present as small dots and more prominently within the nuclei (Figure 3.21, arrows) in the root maturation zone of fully elongated *Arabidopsis* root cells. However, the antibody did not co-localise with the cytoskeletal filaments. There was no distinctive labelling patterns observed in the tobacco BY2 cells (Figure 3.22) or in ghosts (Figure 3.23) labelled with polyclonal anti-caldesmon. Although some labelling was seen in the *Arabidopsis* root cells, the anti caldesmon antibody did not detect any specific bands in either the *Arabidopsis* root extracts or tobacco BY2 extracts (Figure 3.24).

3.4. Actin Filament-Bundling and Cross-Linking Proteins

Antibodies against *Arabidopsis* fimbrin showed extensive labelling in the cytoplasm of *Arabidopsis* root cells, but there was no indication of co-localisation with the actin microfilaments (Figure 3.25). Similarly, anti-fimbrin labelled the cytoplasm of the tobacco BY2 cells but again there was no co-localisation with microfilaments (Figure 3.26) and there was little labelling on ghosts (Figure 3.21). Immunoblotting with the anti-fimbrin antibody detected a distinct band at 73 kDa in *Arabidopsis* root cell extracts, consistent with known molecular weights of the *Arabidopsis* fimbrin proteins (McCurdy and Kim 1998), and a specific band was detected at 64 kDa in the tobacco cell extracts (Figure 3.28).

Extensive patterning was present in the cytoplasm of the *Arabidopsis* root cells labelled with polyclonal plant anti-villin, but again there was no clear correspondence with the actin microfilaments (Figure 3.29). The cytoplasm of tobacco cells also showed extensive labelling with antibodies against plant villin but did not resolve into cytoplasmic structures (Figure 3.30). A single optical section from the stacked image is represented in Figure 3.31 - the labelling pattern was confined exclusively to the cytoplasm and did not resolve into cytoplasmic structures. On ghosts, the level of preservation of actin microfilaments was poor and there was no distinct labelling patterns observed for plant villin (Figure 3.32). A 66 kDa band was observed in the *Arabidopsis* root extracts using the anti-villin antibody in extracts from *Arabidopsis* and two distinct bands at 66 kDa and 34 kDa were observed in tobacco extracts (Figure 3.33). As a faint band at a lower molecular weight of 17 kDa was observed in the tobacco BY2 samples, and as plant villins are known to be greater than 100 kda in size, it is likely that these bands represent proteolytic fragments rather than the whole protein.

Antibodies against animal villin did not recognise specific labelling patterns in the *Arabidopsis* root cells (Figure 3.34 3.27), tobacco BY2 cell (Figure 3.35) or in the ghosts (Figure 3.36). The polyclonal

animal anti-villin antibody identified three bands at 72.5 kDa, 60 kDa and 19 kDa in the tobacco BY2 extracts, a pattern distinct from the labelling pattern observed with the plant-specific antibody, and there were no distinct bands visible in the root samples of *Arabidopsis* (Figure 3.30).

Labelling with polyclonal anti-α-actinin (raised against chicken gizzard protein) detected small dots scattered throughout the root (including in vacuoles) in *Arabidopsis* root cells (Figure 3.37), tobacco BY2 cells (Figure 3.38) and no labelling was present on ghosts (Figure 3.39). Interestingly, a well defined band at 58 kDa was observed in the *Arabidopsis* roots against α-actinin yet no bands were visible in the tobacco BY2 cell extracts (Figure 3.24). α-actinin-like proteins have not previously been reported in plants.

3.5. Cyclase Associated Protein 1

Antibodies against cyclase associated protein 1 (CAP) showed a general cytoplasmic distribution in *Arabidopsis* root cells (Figure 3.40). There were no distinct labelling patterns present in the tobacco BY2 cells (Figure 3.41) or the ghosts (Figure 3.42). The polyclonal anti-cyclase associated protein 1 antibody recognised bands at 55.5 kDa, 98 kDa and a faint band at 69 kDa in the tobacco BY2 extracts and a single band with a molecular weight of 55.5 kDa was seen in *Arabidopsis* root samples (Figure 3.43).The bands seen at ~ 55 kDa in *Arabidopsis* and tobacco BY2 extracts, co-relates to the molecular weight of AtCAP1 from previously published data (~ 50 – 52 kDa)(Chaudhry et al. 2007).

3.6. Capping Proteins

Labelling for α subunit of capping protein (CPA) was generally cytoplasmic in *Arabidopsis* roots, although the antibody also labelled elongated organelle-like structures (Figure 3.44, arrows) consistent with the distended endoplasmic reticulum found in the elongating root cells expressing ER-targeted GFP (Gunning 1998; Haseloff et al. 1997). In tobacco BY2 cells, polyclonal antibodies against α-CPA labelled the membranous areas, notable around the nucleus (Figure 3.45, N) but did not co-localise with the actin cytoskeleton. No distinct labelling patterns were observed in the ghosts for α-CPA (Figure 3.46 A single band was recognised by the polyclonal antibody against α-CPA at a molecular weight of 62 kDa (Figure 3.47). these results were quite different from previously published data, antibodies against AtCPA was detected as a single band of ~39 kDa (Huang et al. 2003).Cells that labelled well with anti-actin also strongly labelled with anti-β-CPB but, there was no distinct co-localisation in the *Arabidopsis* root cells (Figure 3.48). Intriguingly, bands and lines were present in the tobacco BY2 whole cells, including many that matched the thicker actin microfilament bundles (Figure 3.49). Two optical sections from this optical stack showed examples of lines that co-localised with actin microfilament bundles (Figure 3.50B,E, arrows). A second example of co-localisation of β-CPB with microfilament bundles is shown as a stacked image (Figure 3.51, arrows)

and as a single optical section (Figure 3.52, arrows). However, no labelling patterns with polyclonal anti-β-CPB were seen in the ghosts (Figure 3.53). A single band with a molecular weight of 62 kDa was recognised by antibodies against β-CPB in western blotting experiments (Figure 3.48). The molecular weights of this protein was almost twice the molecular weight of previously published data (~ 31 kDa)(Huang et al. 2003).

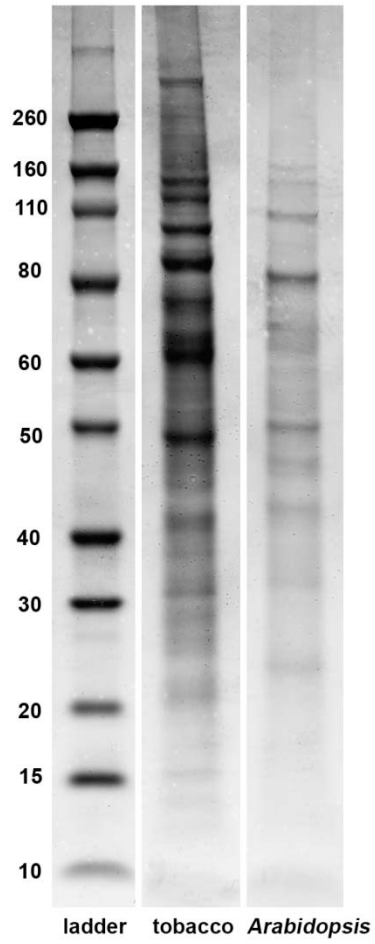

Figure 3.1. SDS gel analysis of *Arabidopsis* roots and tobacco BY2 whole cells extracted with RIPA buffer. Samples were run on a 4 to 12% acrylamide gradient gel and stained with coomassie brilliant blue. Protein concentrations, as measured at 280 nm, were 3.1 mg/ml for the *Arabidopsis* root sample and 2.6 mg/ml for the tobacco BY2 extract.

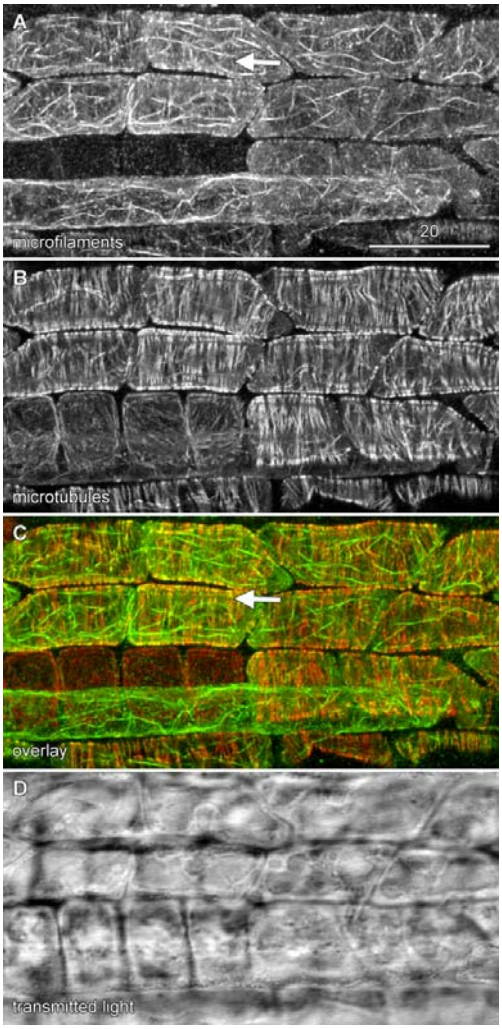

Figure 3.2. The microtubule and actin microfilament cytoskeletons in elongating *Arabidopsis* root cells. Images are confocal images showing approximately 4 optical sections at 1 µm intervals as a maximum projection. Similar reconstructions are shown for subsequent images of *Arabidopsis* root cells. Scale bar in **A** = 20 µm.

A Polyclonal anti-actin labelling of actin microfilaments. The actin is present in two different arrays, including longitudinal bundles and finer, cortical microfilaments that were transversely aligned (arrows).

B Monoclonal anti-tubulin (clone B512) labelling of microtubules. The microtubules were cortical and aligned transverse to the cell axis.

C Overlay - actin microfilaments in green and microtubules in red. In this and subsequent images, the microfilaments are always shown in green no matter the colour of the secondary antibody used.

D Transmitted light.

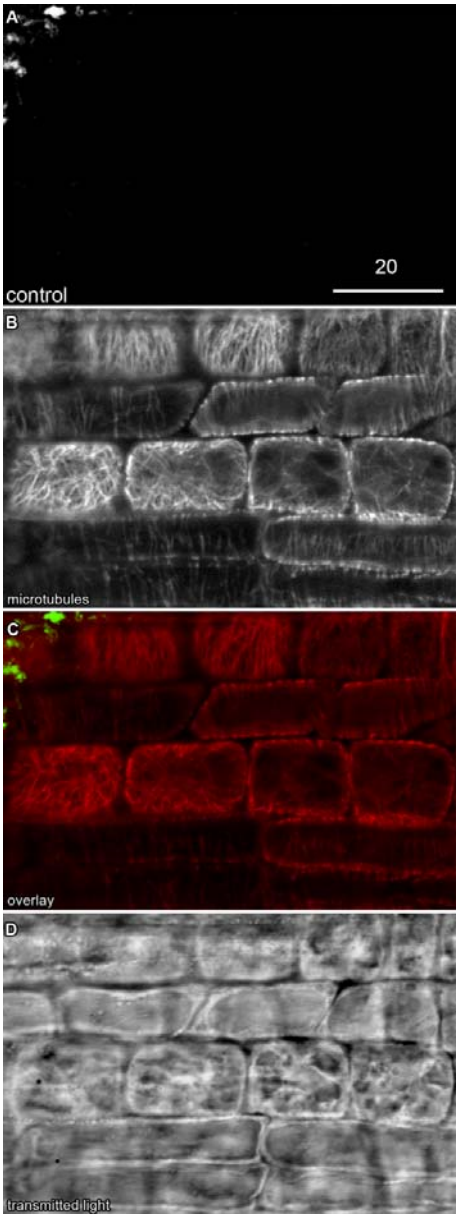

Figure 3.4. Control experiment showing labelling for microtubule filaments only. *Arabidopsis* root cells labelled with only one primary and both the secondaries.

A Microtubules labelled with monoclonal anti-tubulin (clone B512) antibody. The microtubules were cortical and aligned transverse to the cell axis.

B Immunolabelling with both secondary antibodies. No labelling patterns were detected in this emission spectra suggesting that there was no bleed through of signal from the other emission spectra.

C Overlay – actin microfilaments in green

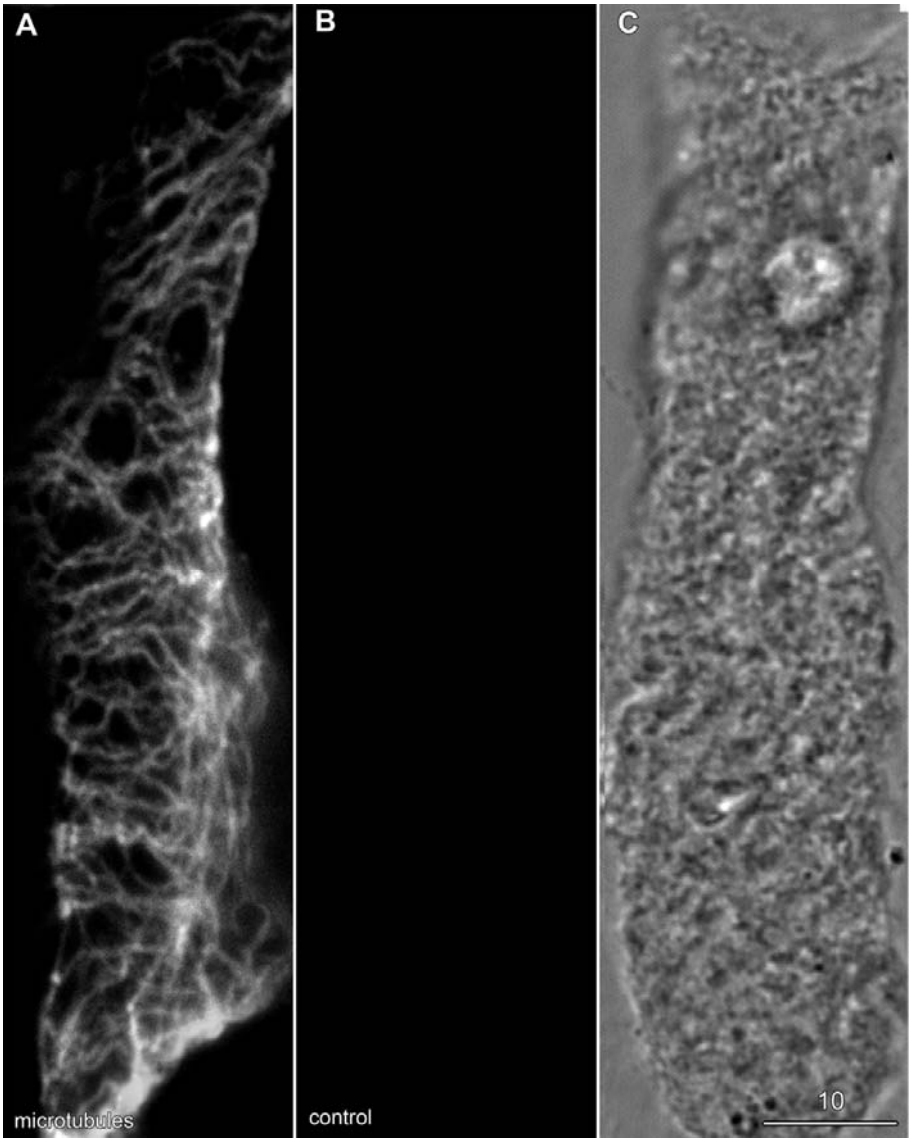

Figure 3.5. The microtubule filament cytoskeleton in tobacco BY2 cells. Images are confocal images showing approximately 4 optical sections at 1 μm intervals as a maximum projection. Similar reconstructions are shown for subsequent images of tobacco BY2 cells except when noted. Cells were labelled only with anti-tubulin antibodies and both the secondary antibodies. This serves as a secondary control experiment to show that there was no cross-talk between emission channels. Scale bar in **C** = 10 μm.

A Monoclonal anti-tubulin (clone B512) labelling of microtubules. The microtubules were cortical and aligned transverse to the cell axis.

B .Secondary control. No labelling indicated that there was no cross-talk between the emission channels.

C Transmitted light.

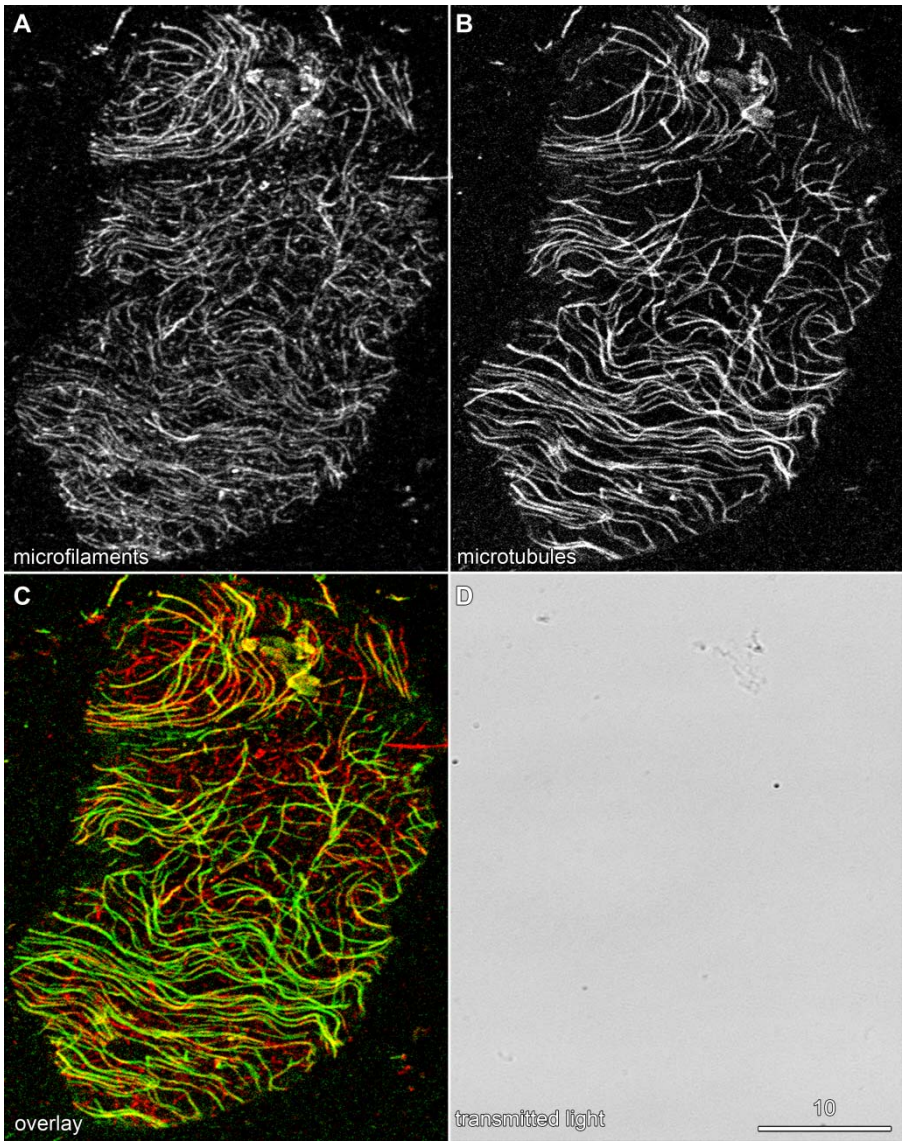

Figure 3.7. Immunolabelling of tobacco BY2 membrane ghosts demonstrated co-localisation of actin microfilaments and microtubules. Immunolabelling results are a single confocal optical section for this and subsequent images of membrane ghosts. A transmitted light image is included in this figure but, as it shows little information, is not included in subsequent figures. Scale bar in **D** = 10 µm.

A Polyclonal anti-actin labelling of actin microfilaments.

B Monoclonal anti-tubulin (clone B512) labelling of microtubules.

C Overlay - actin microfilaments in green and microtubule labelling in red. Some degree of co-alignment is present between the microtubules and microfilaments, as demonstrated by yellow labelling in the overlay, but examples are also present of non-co aligned microtubules and microfilaments.

D Transmitted light. As only the plasma membrane is present, little can be discerned in transmitted light images of ghosts.

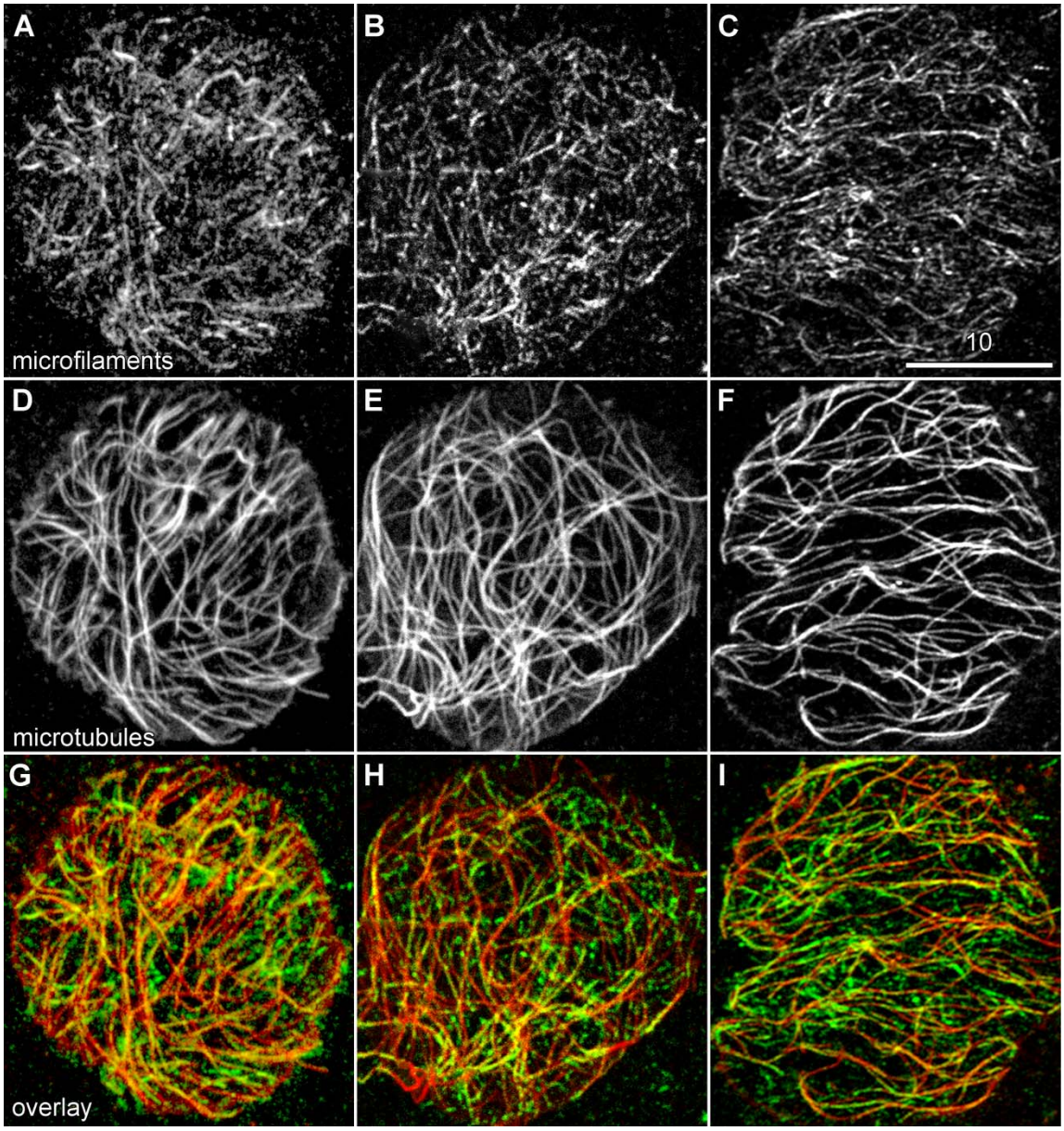

Figure 3.8. Immunolabelling of 3 replicate membrane ghosts with polyclonal anti-actin and monoclonal anti-tubulin. Scale bar in **C** = 10 μm.

A,B,C Polyclonal anti-actin labelling of microfilaments.

D,E,F Anti-tubulin.

G,H,I Overlay - actin microfilaments in green and microtubule labelling in red. The levels of co-localisation and co-alignment vary between ghosts.

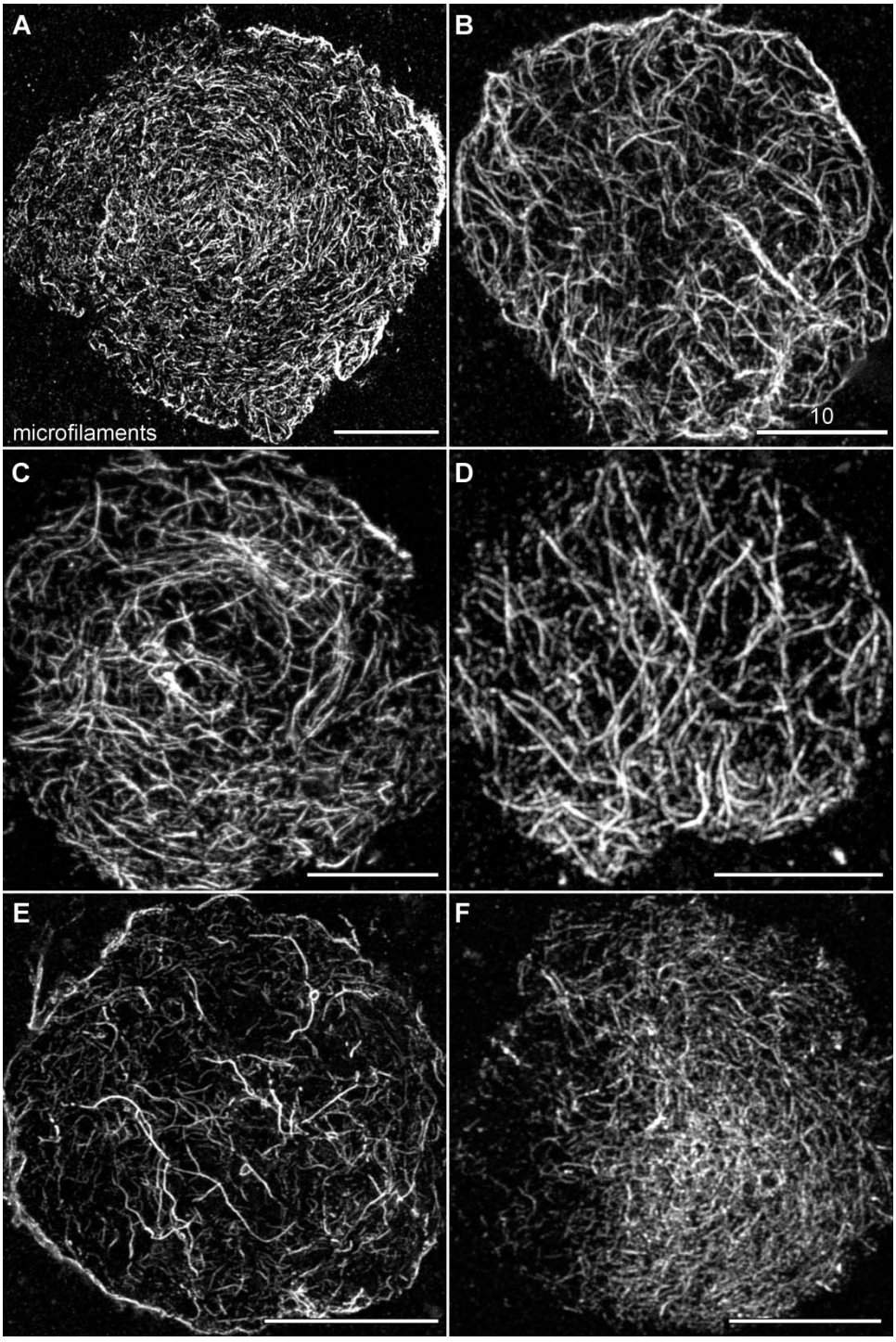

Figure 3.9. Replicate images of actin microfilaments on membrane ghosts labelled with monoclonal anti-actin. Patterns in **A**-**F** were similar to microfilaments observed with polyclonal anti-actin. Scale bar in **B** = 10 μm.

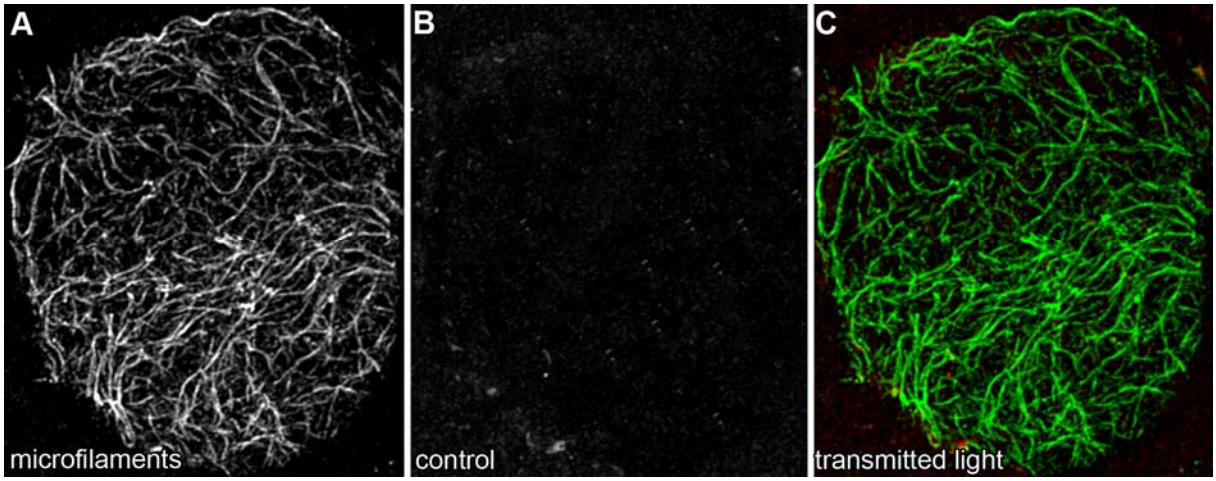

Figure 3.10 Labelling ghosts with only one primary antibody and both the secondaries. Control experiment to show that there was minimal crosstalk between emission spectra. Scale bar in **C** = 10 µm.

A Microfilament labelling with polyclonal anti actin.

B No labelling seen in this emission channel.

C Transmitted light.

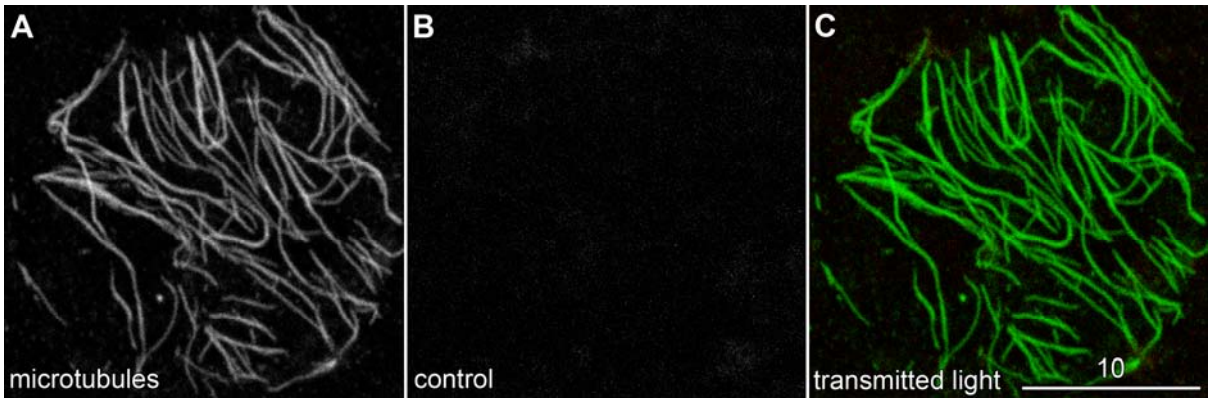

Figure 3.11 Labelling ghosts with only one primary antibody and both the secondaries. Control experiment to show that there was minimal crosstalk between emission spectra. Scale bar in **C** = 10 µm.

A Microtubule filament labelling with monoclonal anti tubulin (clone B512).

B No labelling seen in this emission channel.

C Transmitted light.

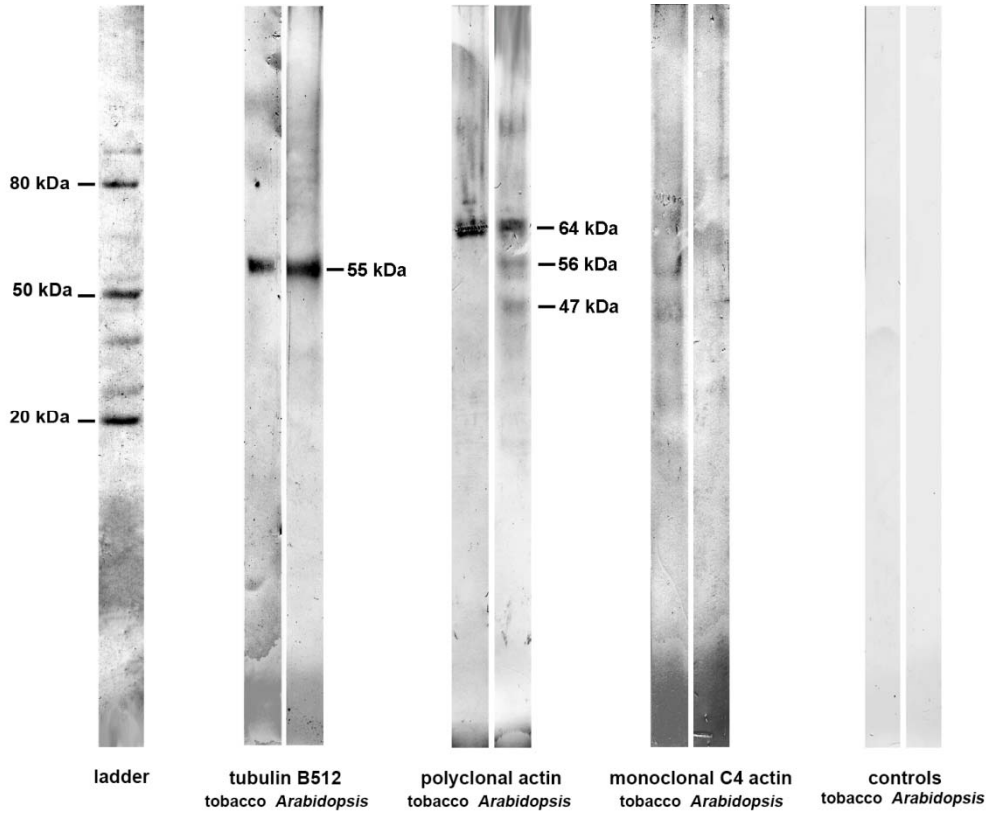

Figure 3.12. Western blotting analysis of actin and tubulin in *Arabidopsis* root cells and tobacco BY2 whole cells. The secondary controls in this image are for both the secondary antibodies (mouse and rabbit).

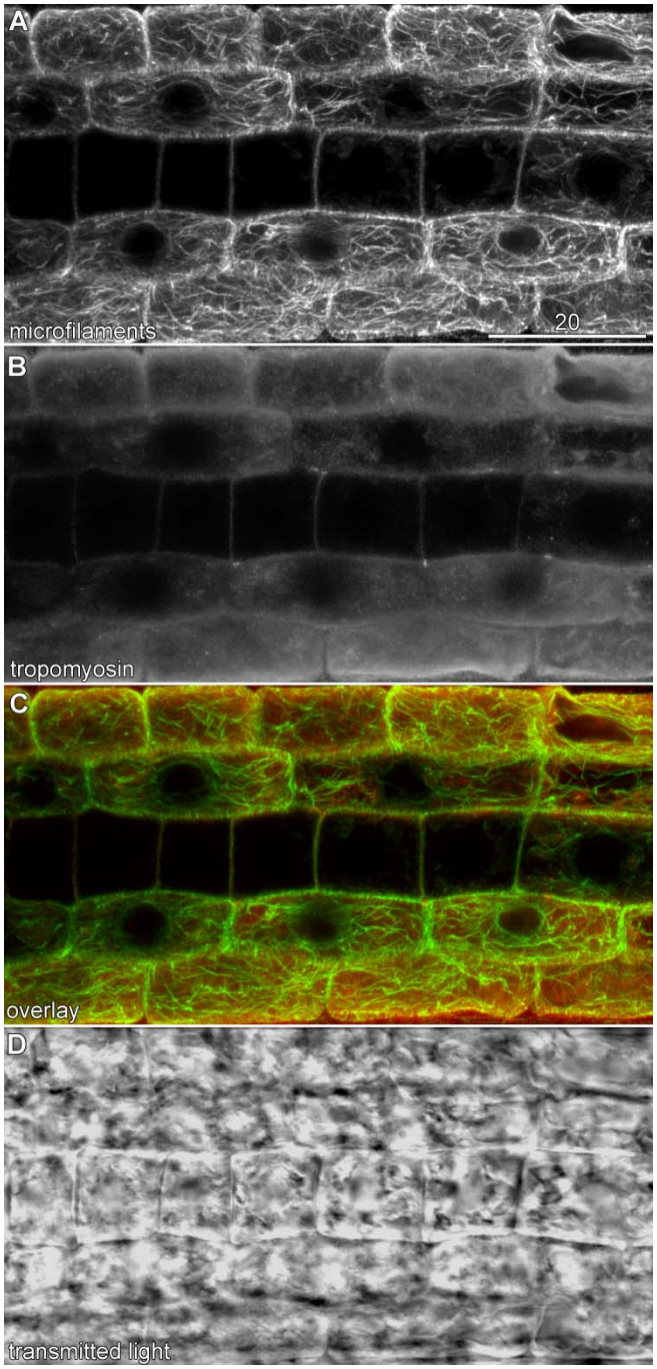

Figure 3.13. Immunolabelling of *Arabidopsis* root cells with anti-actin and polyclonal anti-tropomyosin. Scale bar in **A** = 20 µm.

A Monoclonal anti-actin labelling of microfilaments

B Anti-tropomyosin. No distinct labelling was observed in elongating cells.

C Overlay – actin microfilaments in green and tropomyosin labelling in red.

D Transmitted light.

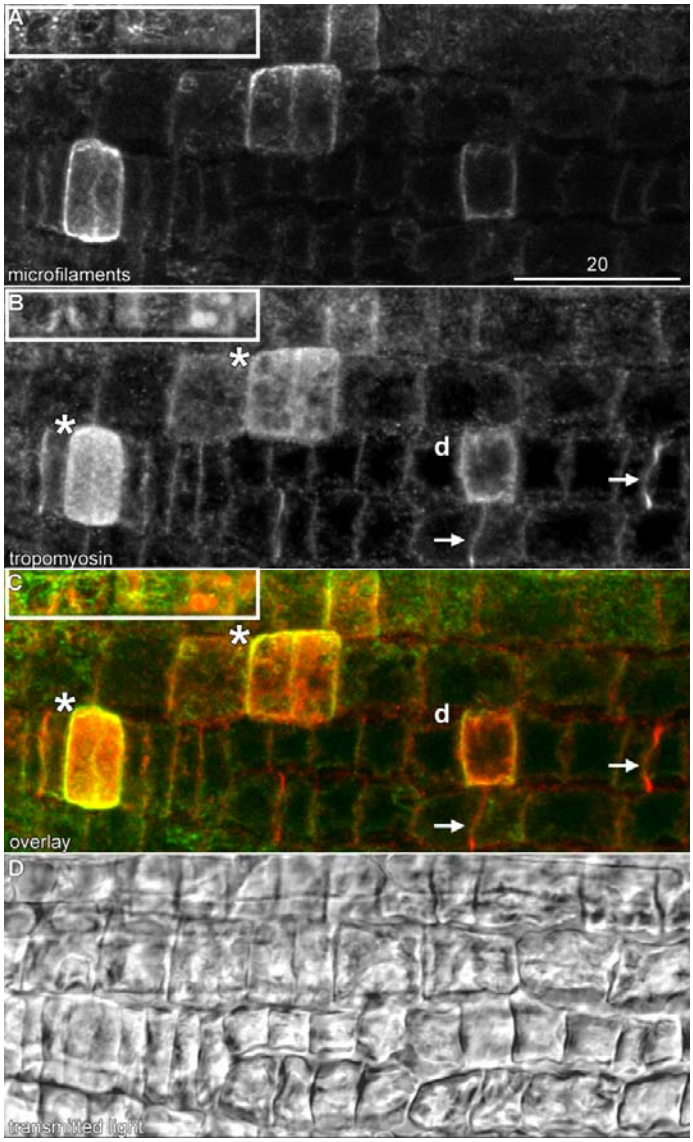

Figure 3.14. Immunolabelling of *Arabidopsis* root cells with polyclonal anti-tropomyosin. Scale bar in **A** = 20 µm.

A Monoclonal anti-actin labelling of microfilaments. The periphery of dividing cells were clearly labelled with the actin antibody (*, asterisks).

B Anti-tropomyosin. Dividing cells labelled with the tropomyosin antibody (*, asterisks), but newly formed cell walls and developing plates were also labelled (arrows). A cell in its last stages of dividing (d), tropomyosin is localised mainly to the cell wall and edges of cytoplasm. That the tropomyosin signal was not cross – talk from the microfilament labelling is demonstrated by the boxed region in which the tropomyosin patterning is clearly different from the actin microfilament labelling shown in panel **A.**

C Overlay – actin microfilaments in green and tropomyosin labelling in red.

D Transmitted light

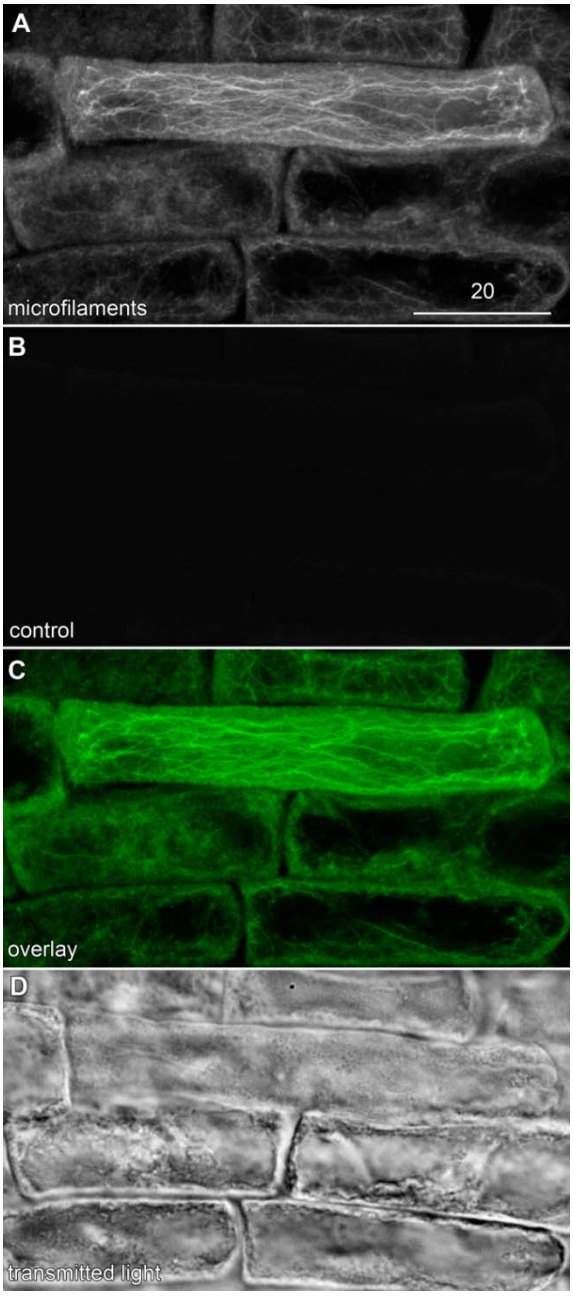

Figure 3.15 Actin microfilament control with both the secondary antibodies. Only a single primary antibody was used for labelling the cells with both the secondary antibodies. Secondary antibodies were specific to the primary antibodies. Scale bar in **A** = 10 µm.

A Actin microfilaments labelled with monoclonal C4 anti-actin antibody. Thick filament bundles running longitudinal to the cell axis and finer cortical microfilaments were identified.

B Immunolabelling with both secondary antibodies. No labelling patterns were detected in this emission spectra suggesting that there was no bleed through of signal from the other emission spectra.

C Overlay – actin microfilaments in green

D Transmitted light

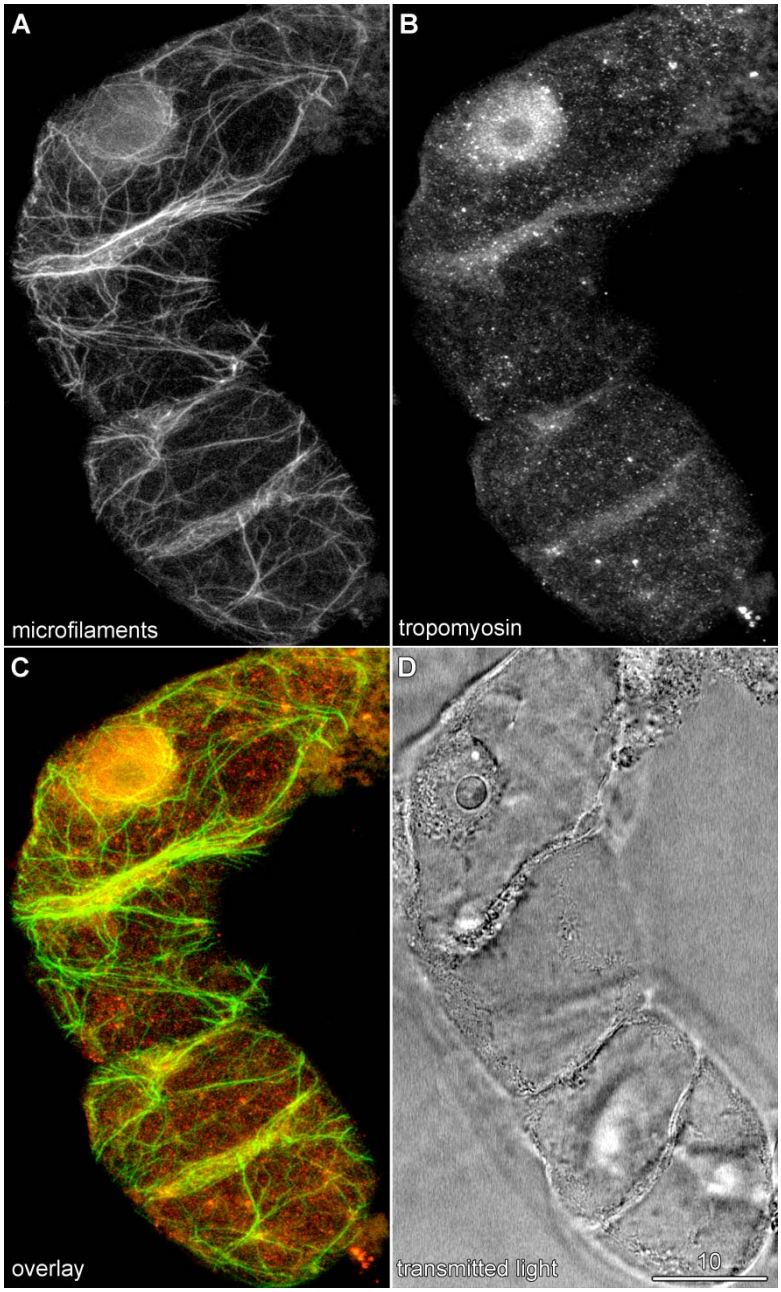

Figure 3.16. Immunolabelling of tobacco BY2 cells with polyclonal anti-tropomyosin. Scale bar in **D** = 10 μm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-tropomyosin labelling showed no distinct association with cellular structures. In contrast to *Arabidopsis* root cells, no evidence was seen for preferential anti-tropomyosin localisations associated with dividing cells or newly formed cell wall.

C Overlay - actin microfilaments in green and tropomyosin in red. In this and subsequent images, the microfilaments are always shown in green no matter the colour of the secondary antibody used

D Transmitted light.

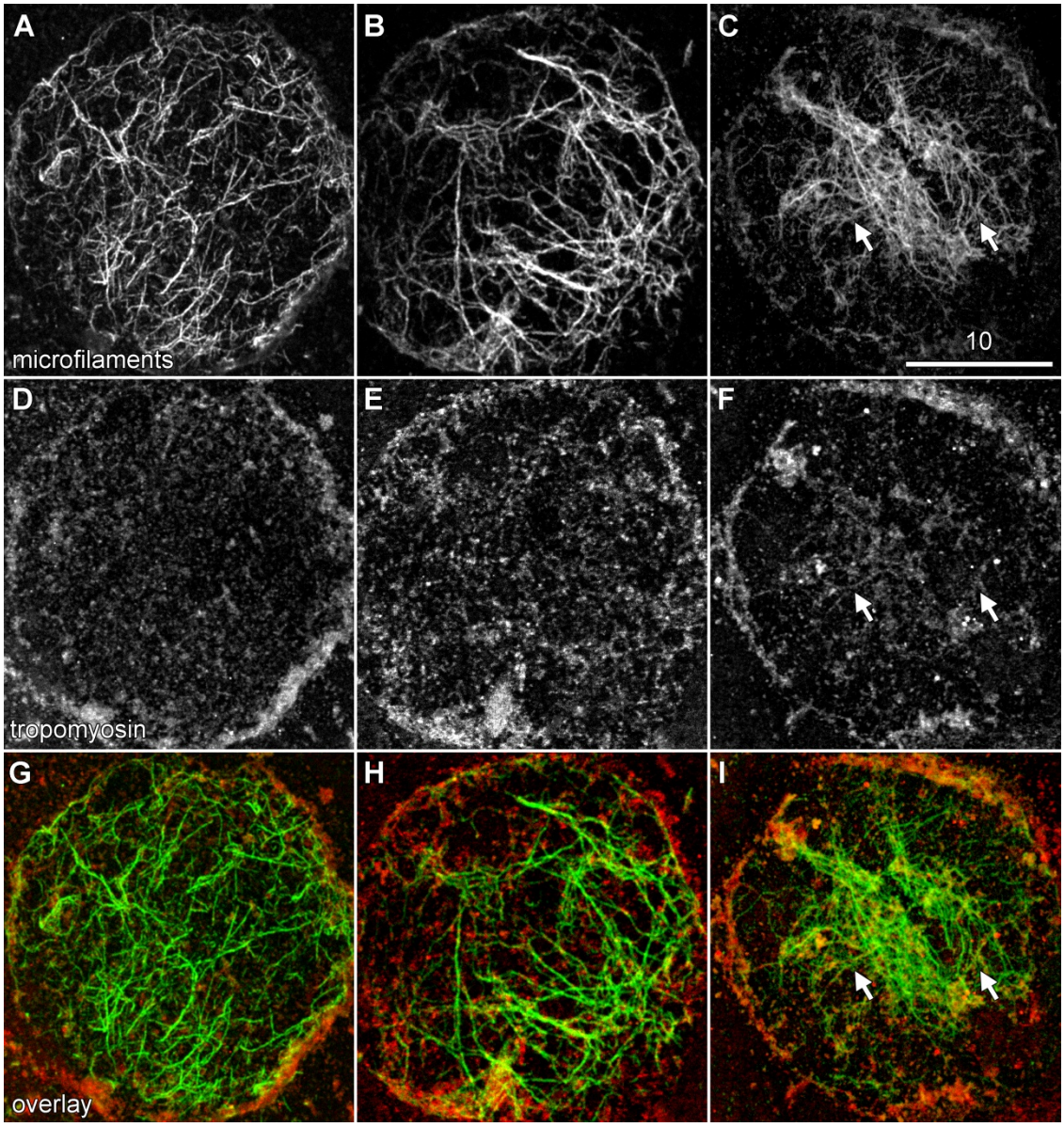

Figure 3.17. Immunolabelling of 3 membrane ghosts with polyclonal anti-tropomyosin. Scale bar in **C** = 10 μm.

A,B,C Monoclonal anti-actin labelling of microfilaments.

D,E,F Anti-tropomyosin. It is tempting to speculate that lines are present within some images of tropomyosin labelled ghosts, but no widespread co-localisation with microfilaments was present. Examples where co-localisation does appear to be present in panel **F** are highlighted with arrows.

G,H,I Overlay - actin microfilaments in green and tropomyosin labelling in red.

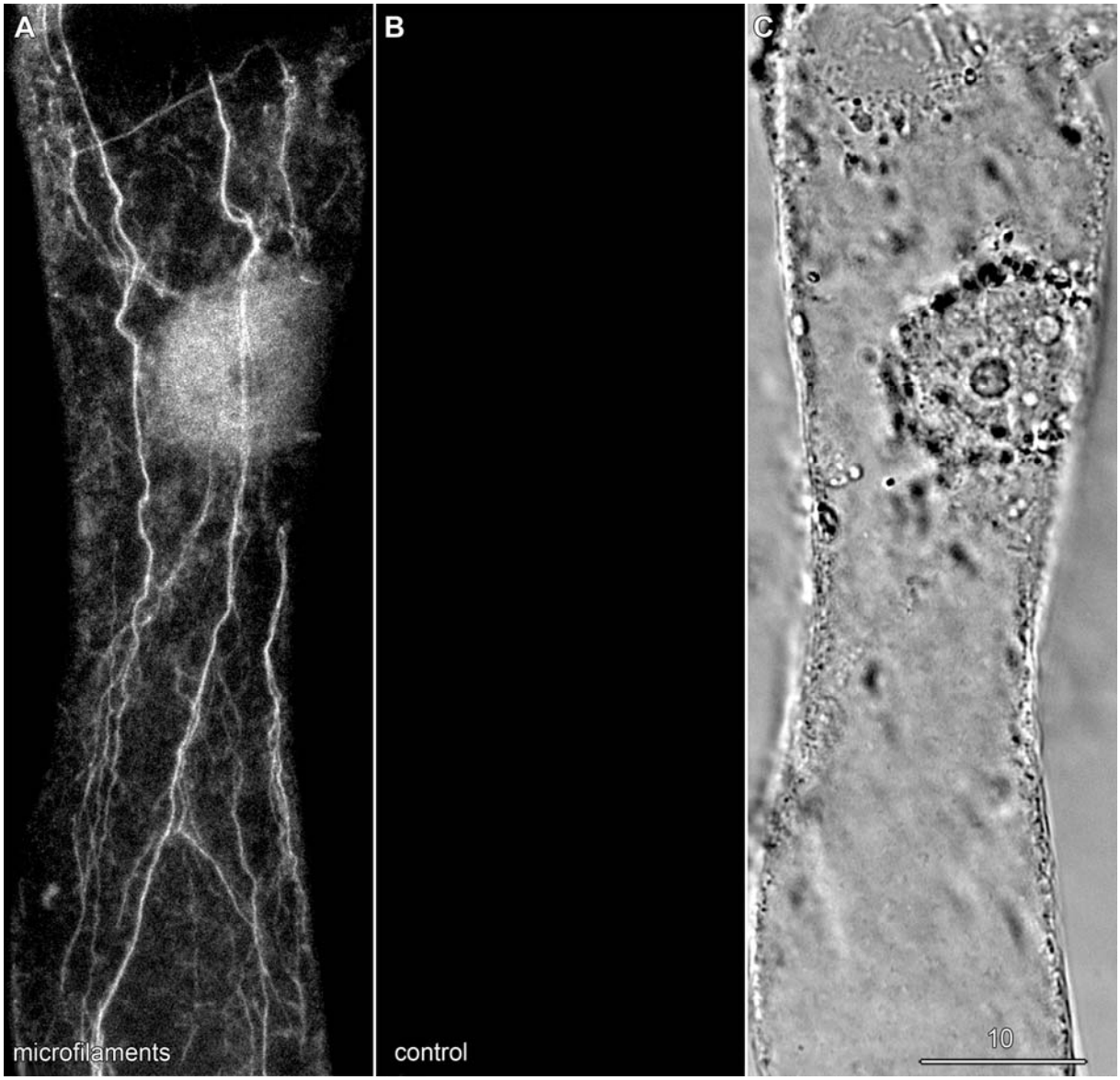

Figure 3.18. Immunolabelling of actin microfilament cytoskeleton in tobacco BY2 cells. the cells were labelled with both the secondaries with only the monoclonal antibody against actin. Scale bar in **C** = 10 μm.

A Monoclonal C4 anti-actin labelling of microtubules. Microfilaments were seen as thick bundles running longitudinal to the cell axis and some were seen as finer cortical transverse filaments.

B .Secondary control. No labelling indicated that there was no cross-talk between the emission channels.

C Transmitted light

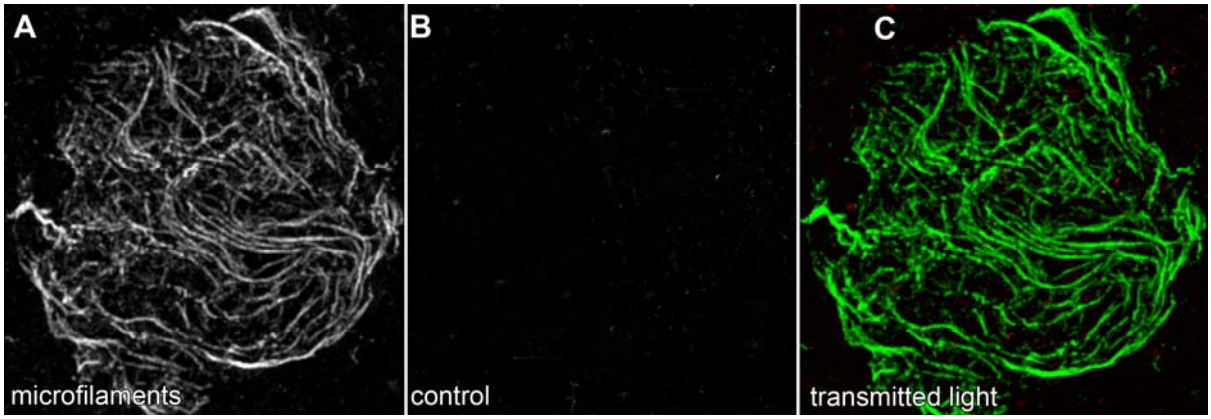

Figure. 3.19 Labelling ghosts with only one primary antibody and both the secondaries. Control experiment to show that there was minimal crosstalk between emission spectra. Scale bar in **C** = 10 µm.

A Microfilament labelling with monoclonal C4 anti actin.

B No labelling seen in this emission channel.

C Transmitted light.

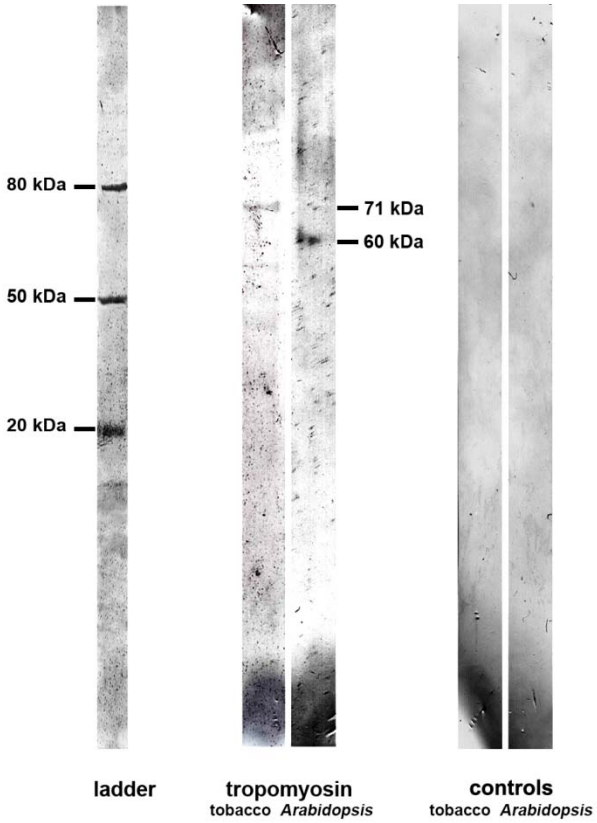

Figure 3.20. Western blotting analysis of tropomyosin in *Arabidopsis* root cells and tobacco BY2 whole cells.

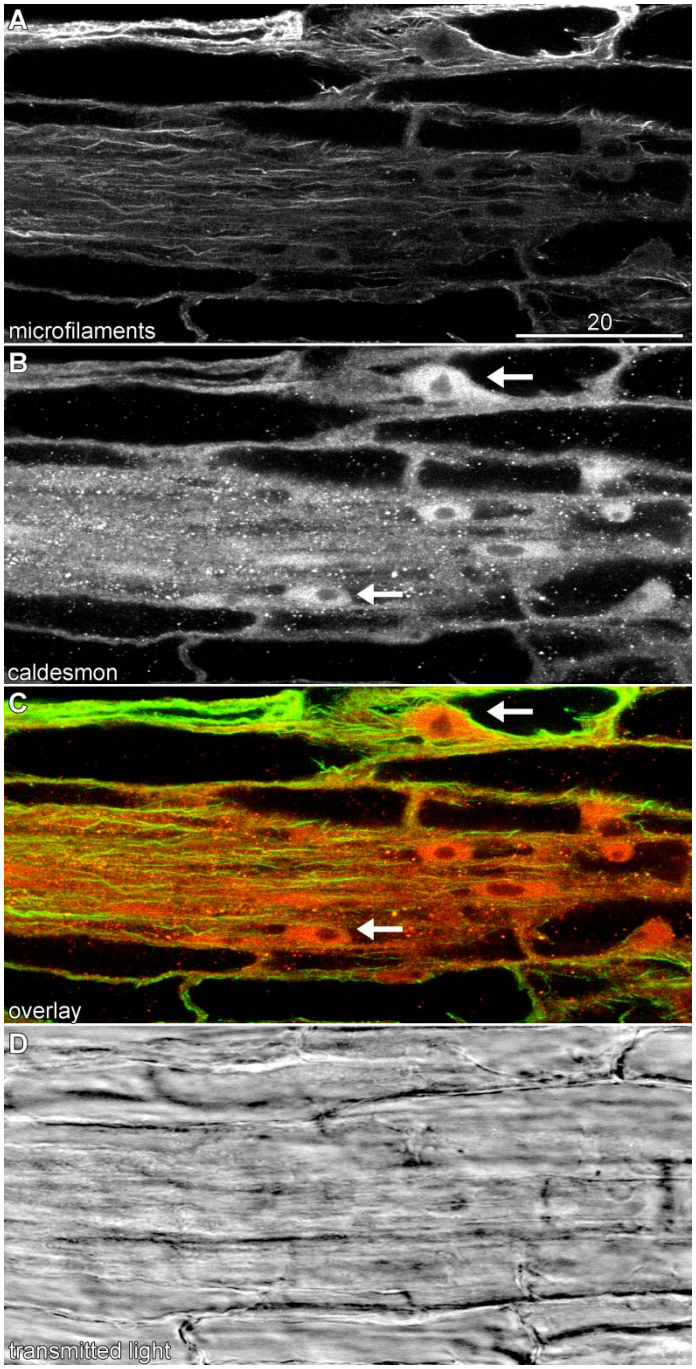

Figure 3.21. Immunolabelling of fully elongated *Arabidopsis* root cells from the root maturation zone with polyclonal anti-caldesmon. Scale bar in **A** = 20 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-caldesmon labelling was present as small dots, but was also prominent within the nuclei (arrows). No cytoskeletal associations were seen.

C Overlay - actin microfilaments in green and caldesmon labelling in red.

D Transmitted light.

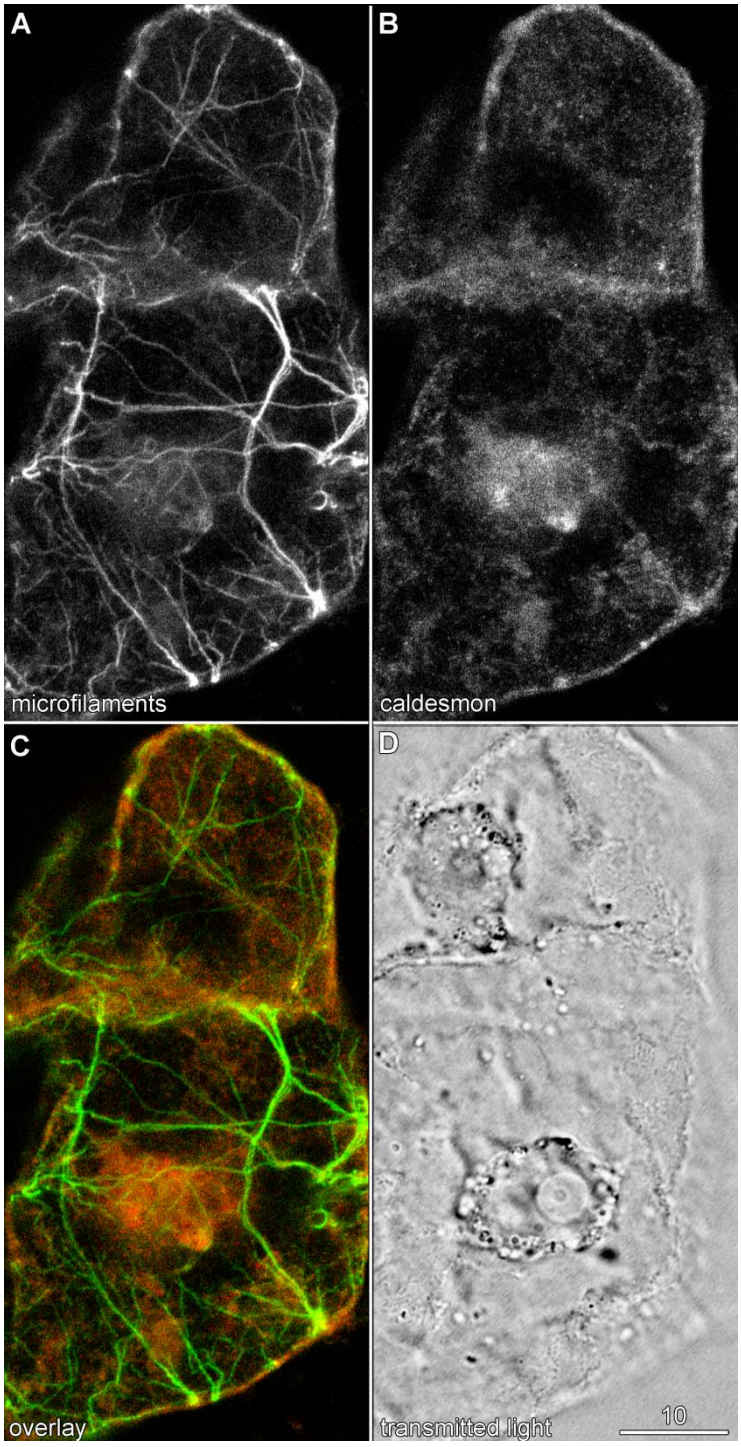

Figure 3.22. Immunolabelling of tobacco whole cells polyclonal anti-caldesmon. Scale bar in **D** = 10 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-caldesmon. Little labelling was present.

C Overlay - actin microfilaments in green and caldesmon labelling in red.

D Transmitted light.

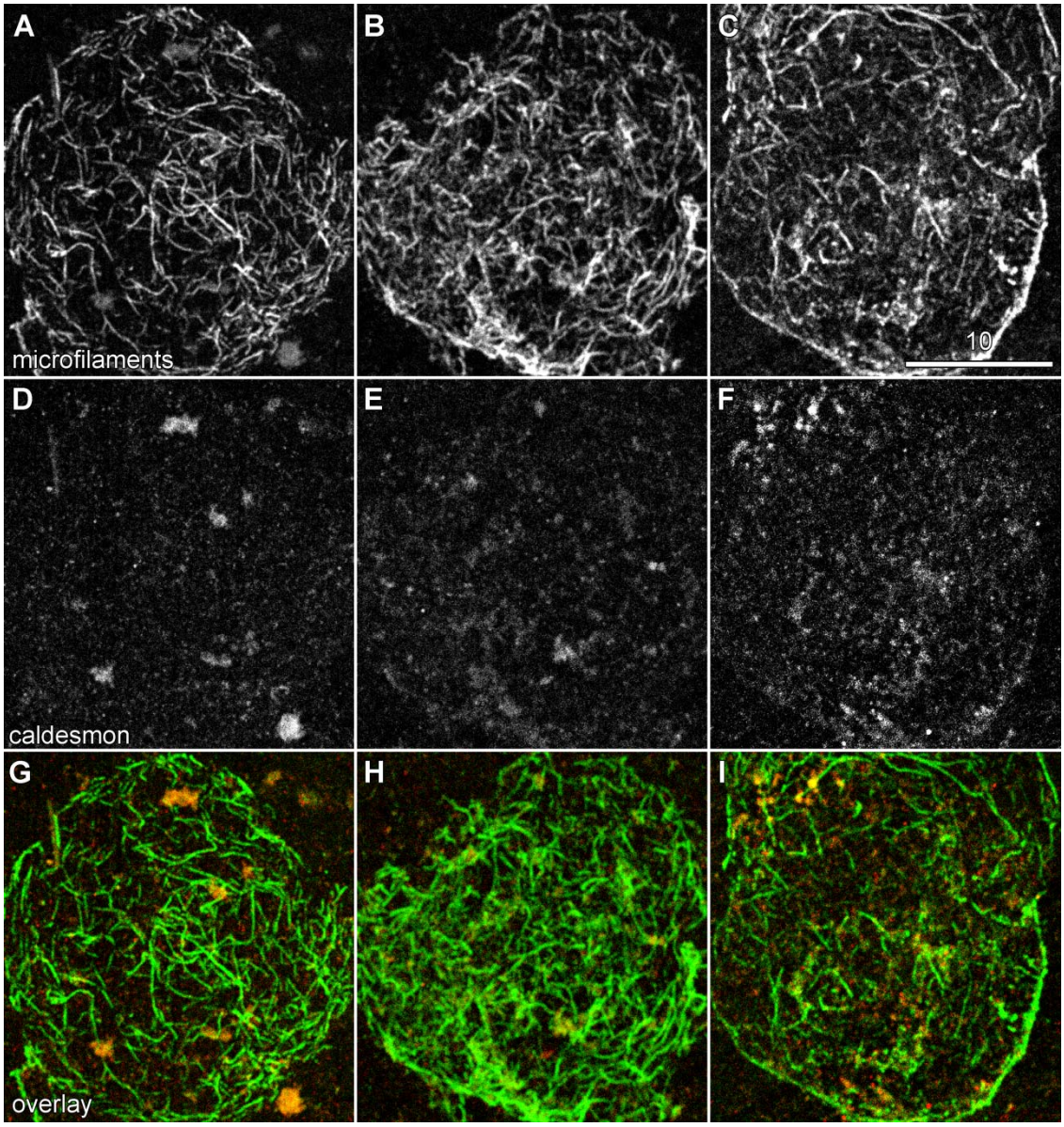

Figure 3.23. Immunolabelling of 3 membrane ghosts made with polyclonal anti-caldesmon. Scale bar in **C** = 10 μm.

A,B,C Monoclonal anti-actin labelling of microfilaments.

D,E,F Anti-caldesmon. No labelling of ghosts was present.

G,H,I Overlay - actin microfilaments in green and caldesmon labelling in red.

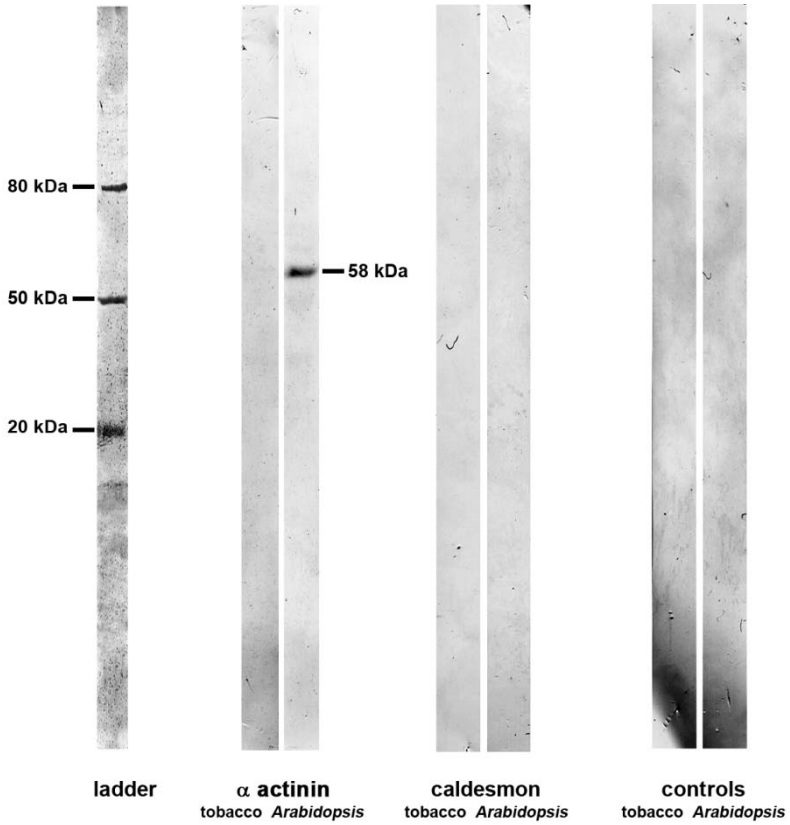

Figure 3.24. Western blotting analysis of α actinin and caldesmon in *Arabidopsis* root cells and tobacco BY2 whole cells.

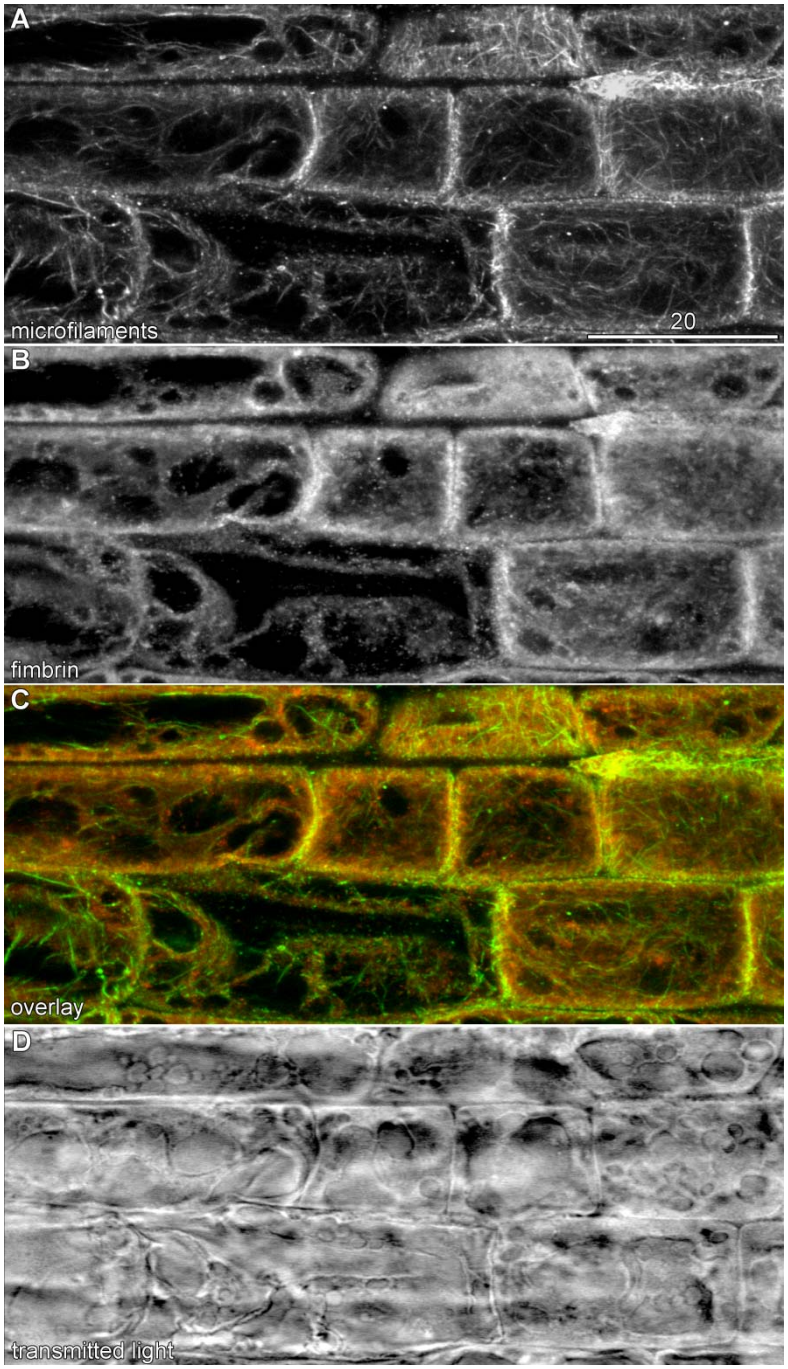

Figure 3.25. Immunolabelling of elongating *Arabidopsis* root cells with polyclonal anti-fimbrin. Scale bar in **A** = 20 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-fimbrin labelling in the cytoplasm was extensive but showed no co-localisation with the microfilaments.

C Overlay - actin microfilaments in green and fimbrin labelling in red.

D Transmitted light.

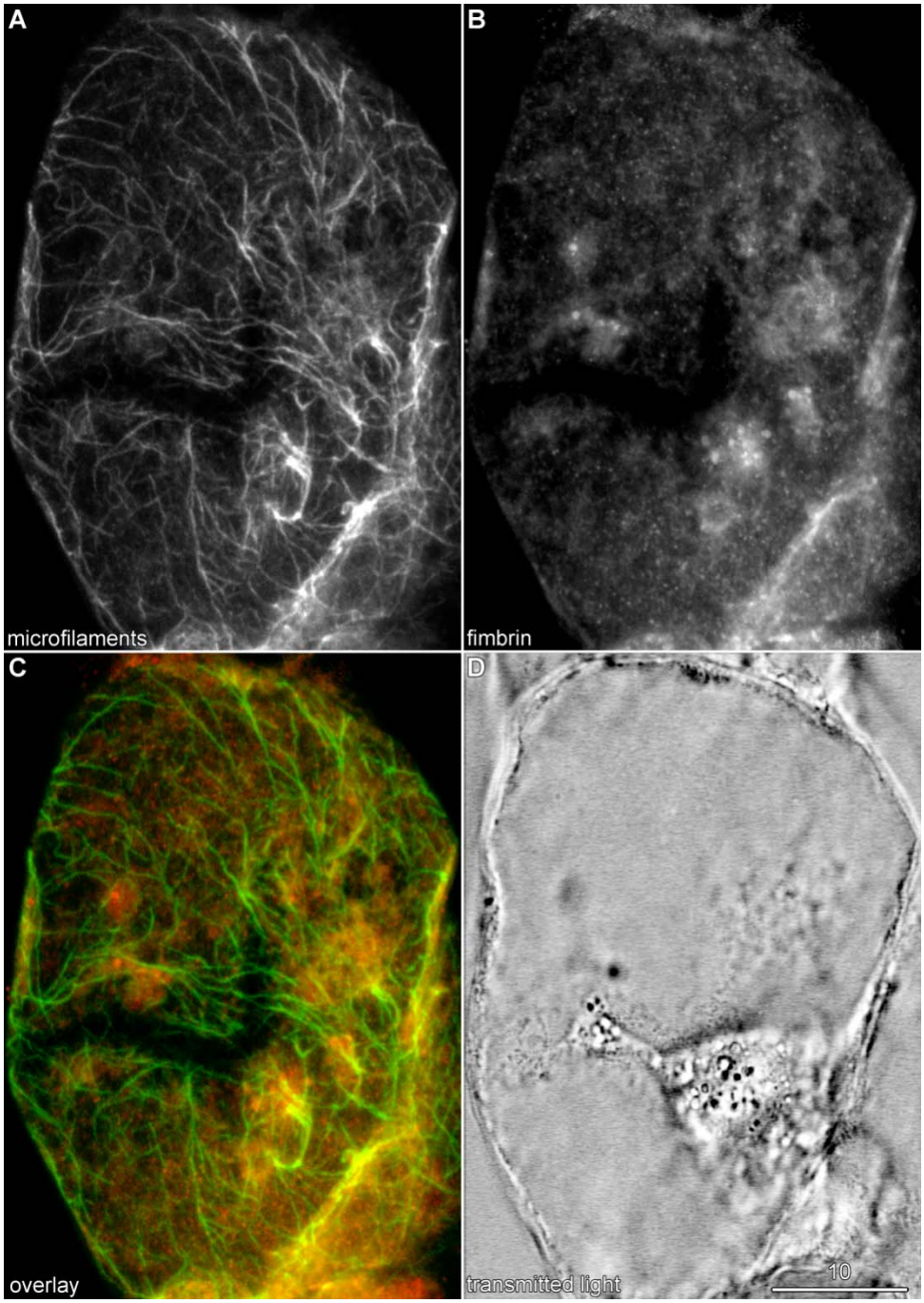

Figure 3.26. Immunolabelling of tobacco BY2 cells with polyclonal anti-fimbrin. Scale bar in **D** = 10 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-fimbrin labelling was exclusively cytoplasmic.

C Overlay - actin microfilaments in green and fimbrin labelling in red.

D Transmitted light.

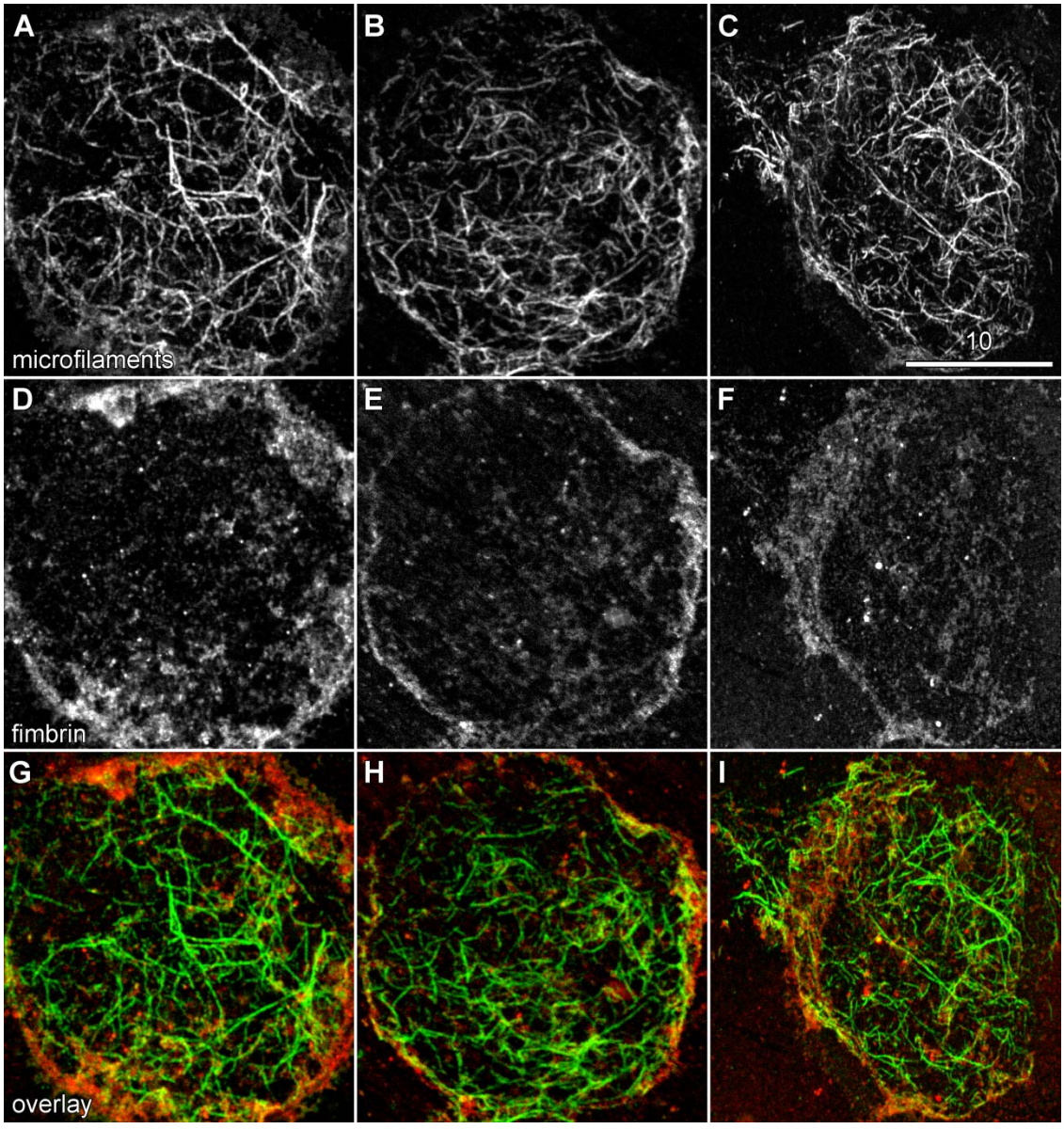

Figure 3.27. Immunolabelling of 3 membrane ghosts with polyclonal anti-fimbrin. Scale bar in **C** = 10 µm.

A,B,C Monoclonal anti-actin labelling of microfilaments.

D,E,F Anti-fimbrin. Ghosts showed little labelling.

G,H,I Overlay - actin microfilaments in green and fimbrin labelling in red.

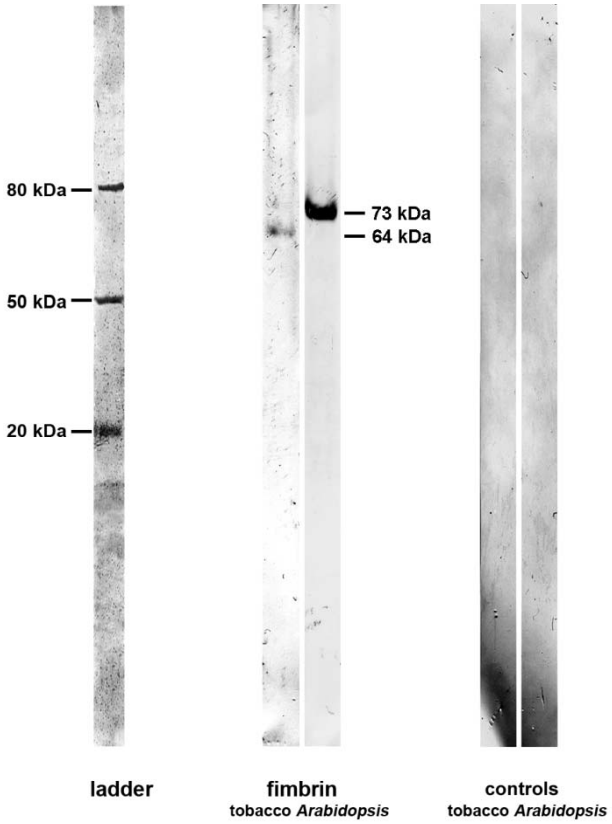

Figure 3.28. Western blotting analysis of fimbrin in *Arabidopsis* root cells and tobacco BY2 whole cells.

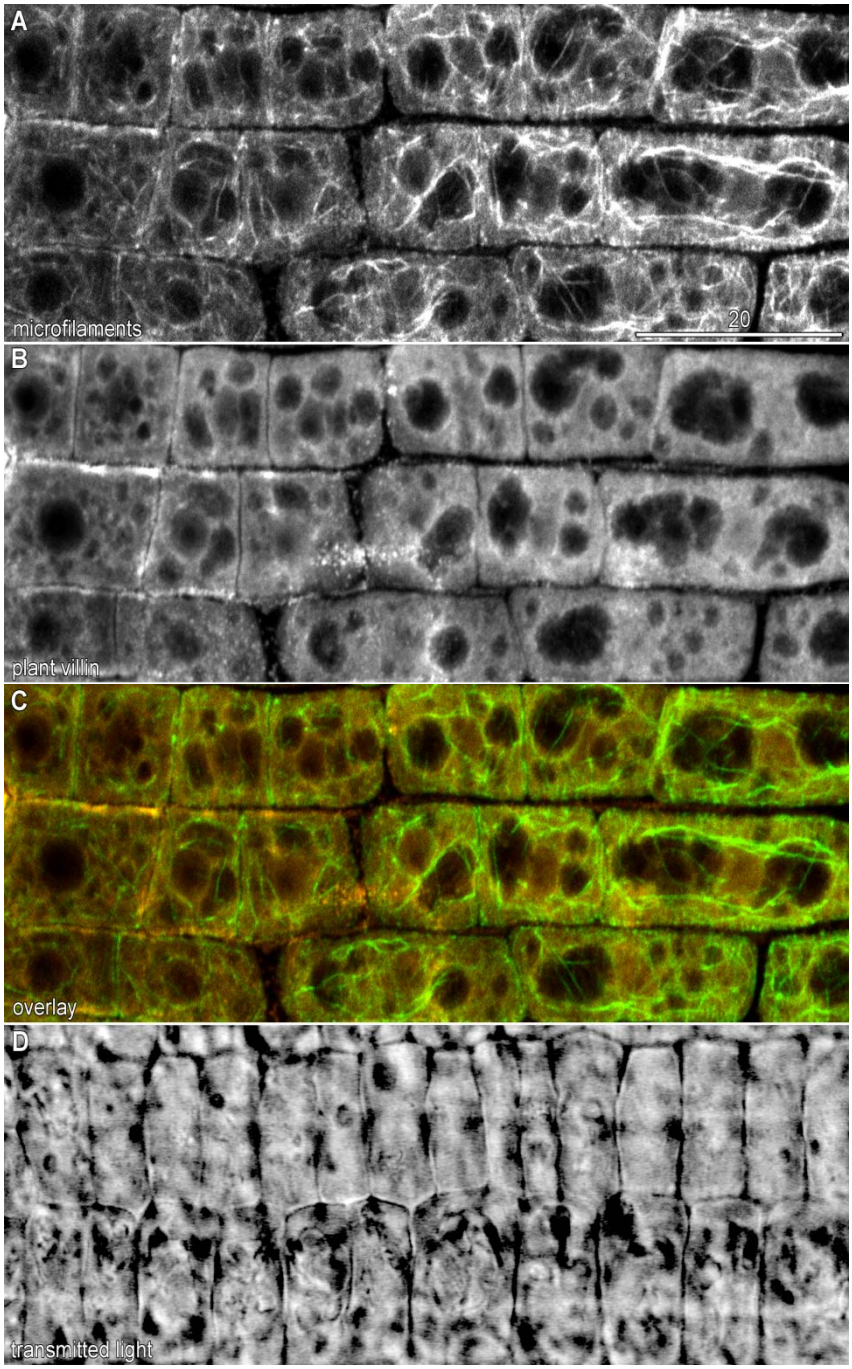

Figure 3.29. Immunolabelling of elongating *Arabidopsis* root cells with polyclonal plant anti-villin. Scale bar in **A** = 20 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Plant anti-villin: although some patterning was present within the cytoplasm, no clear correspondence was present with the actin microfilaments.

C Overlay - actin microfilaments in green and villin labelling in red.

D Transmitted light.

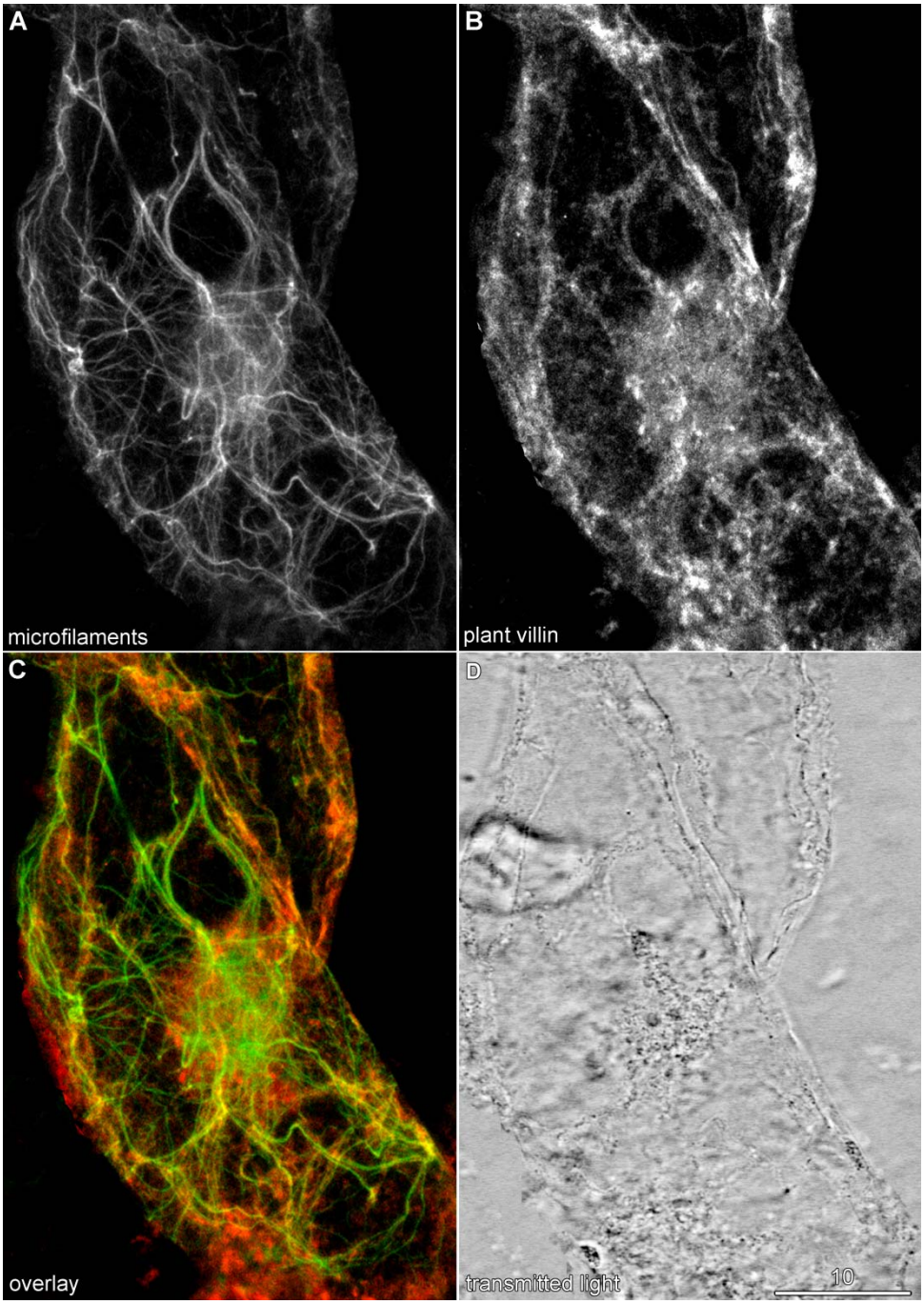

Figure 3.30. Immunolabelling of tobacco BY2 cells with polyclonal plant anti-villin. Scale bar in **D** = 10 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Antibodies against plant villin gave extensive cytoplasmic labelling but did not resolve into cytoplasmic structures.

C Overlay - actin microfilaments in green and anti-plant villin labelling in red.

D Transmitted light.

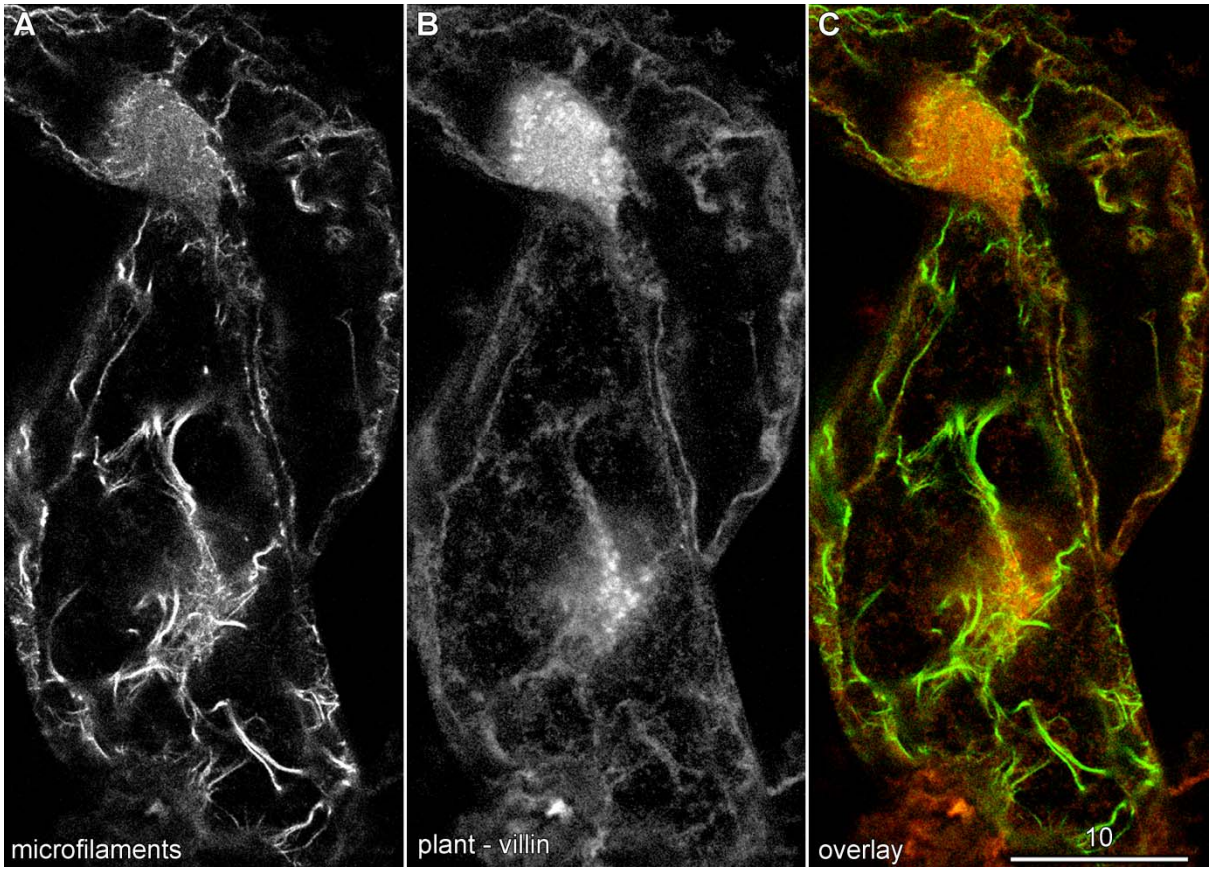

Figure 3.31. A single optical section showing anti-plant villin labelling in tobacco BY2 cells. Scale bar in **C** = 10 μm.

A Monoclonal anti-actin labelling of microfilaments.

B Plant anti-villin. Labelling is exclusively cytoplasmic, although distinct cytoskeletal structures are not visible.

C Overlay - actin microfilaments in green and anti-plant villin labelling in red.

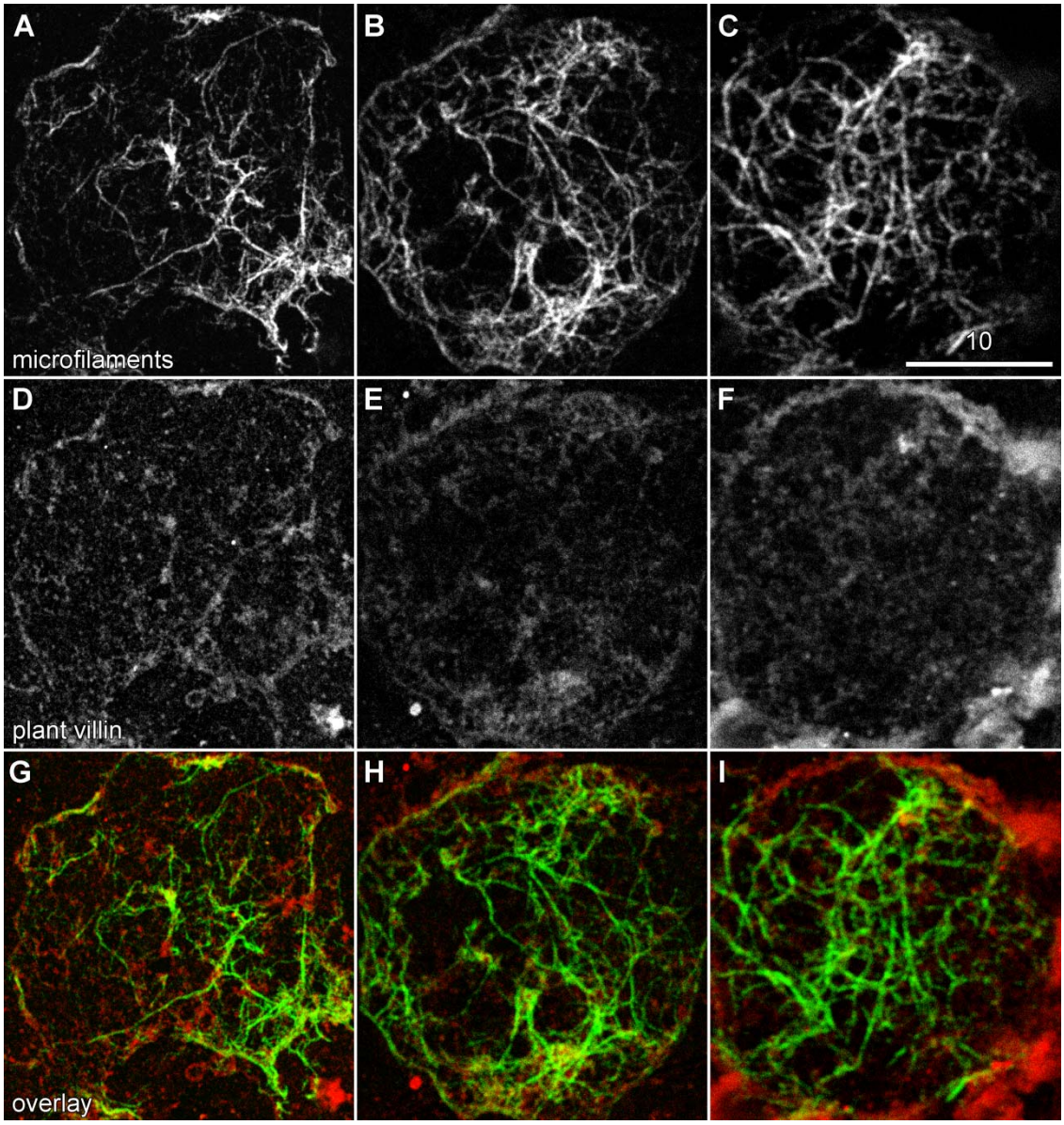

Figure 3.32. Immunolabelling of 3 membrane ghosts with polyclonal plant anti-villin. Scale bar in **C** = 10µm.

A,B,C Monoclonal anti-actin labelling of microfilaments. The level of microfilament preservation on these ghosts was poor.

D,E,F Anti-plant villin. Ghosts showed little labelling.

G,H,I Overlay - actin microfilaments in green and plant villin labelling in red.

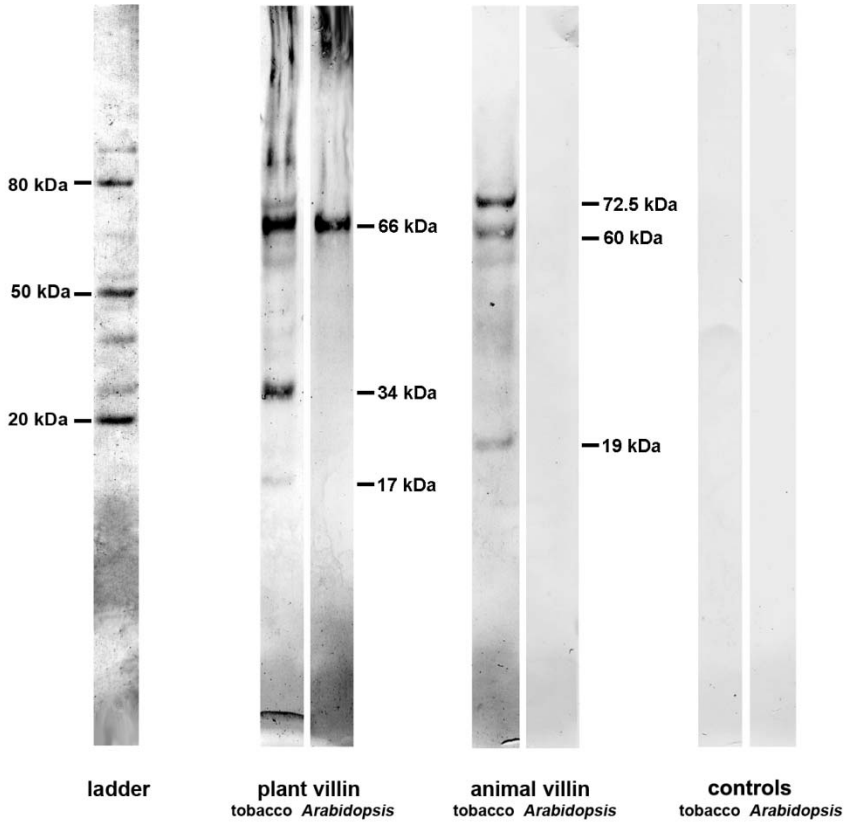

Figure 3.33. Western blotting analysis of villin in *Arabidopsis* root cells and tobacco BY2 whole cells.

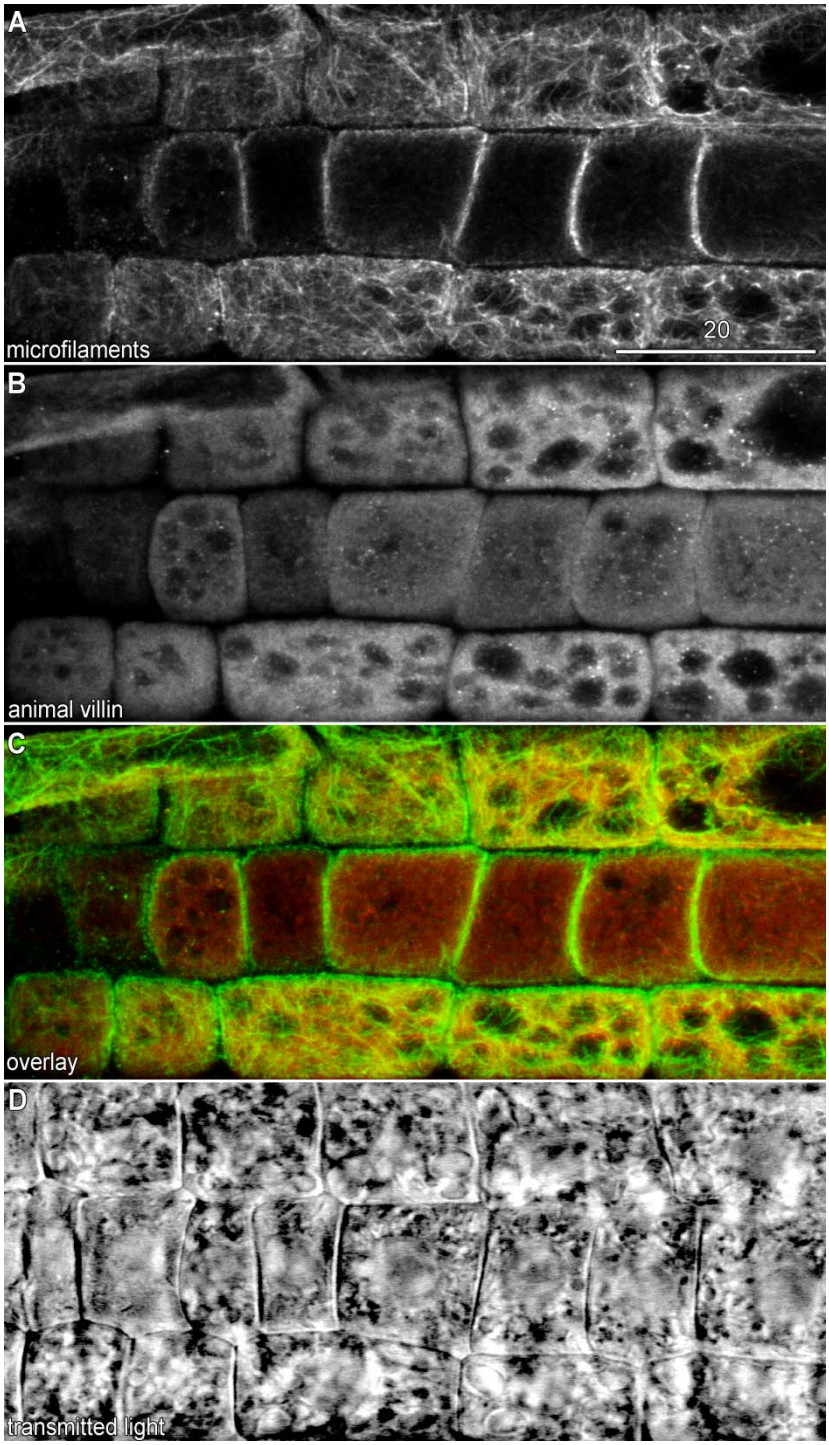

Figure 3.34. Immunolabelling of elongating *Arabidopsis* root cells with polyclonal animal anti-villin. Scale bar in **A** = 20 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Animal anti-villin - no distinct labelling was observed with this antibody.

C Overlay - actin microfilaments in green and villin labelling in red.

D Transmitted light.

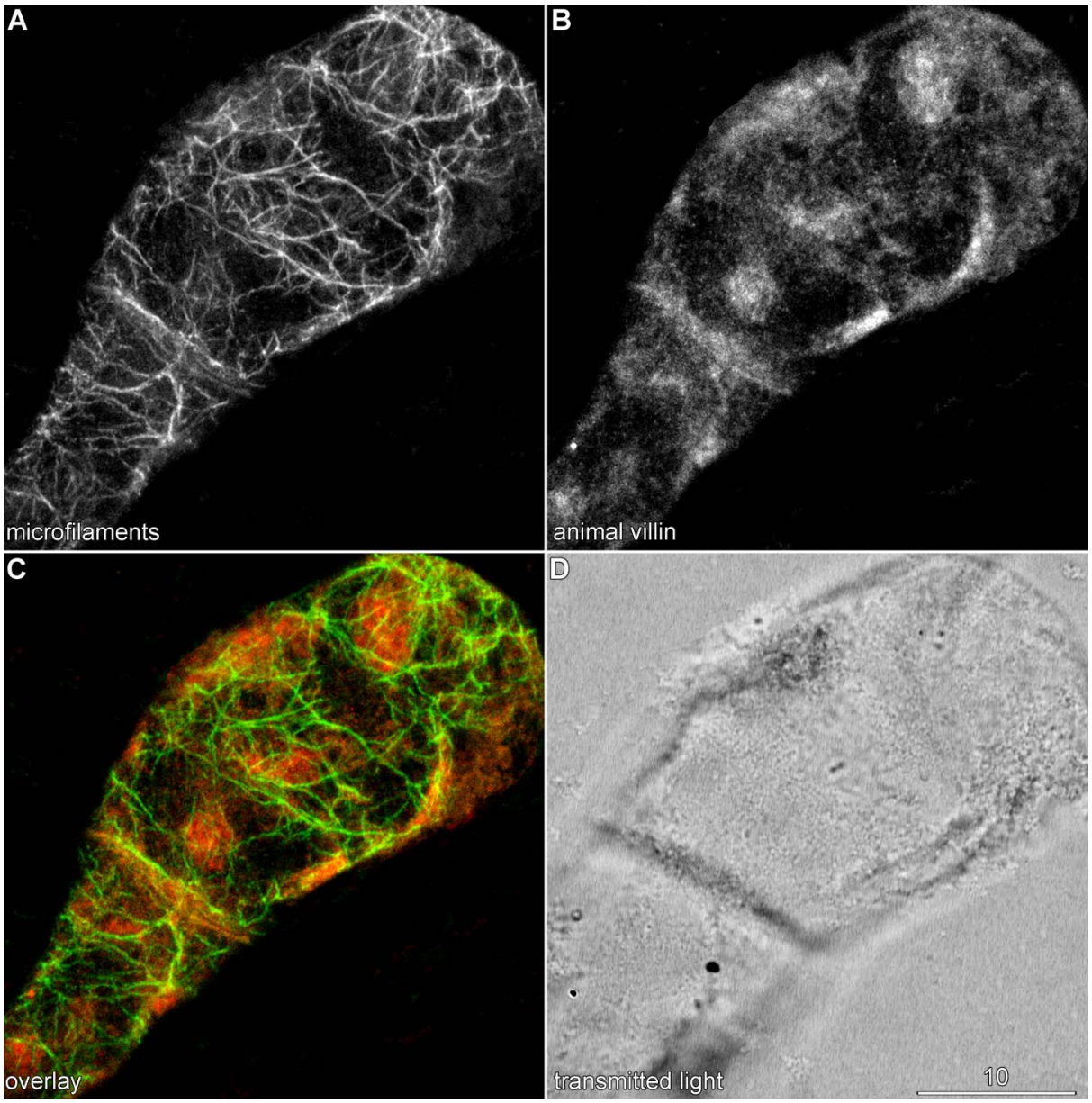

Figure 3.35. Immunolabelling of tobacco BY2 cells with polyclonal animal anti-villin. Scale bar in **D** =10 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Animal anti-villin gave a general cytoplasmic labelling with no suggestion of association with the cytoskeleton.

C Overlay - actin microfilaments in green and villin labelling in red.

D Transmitted light.

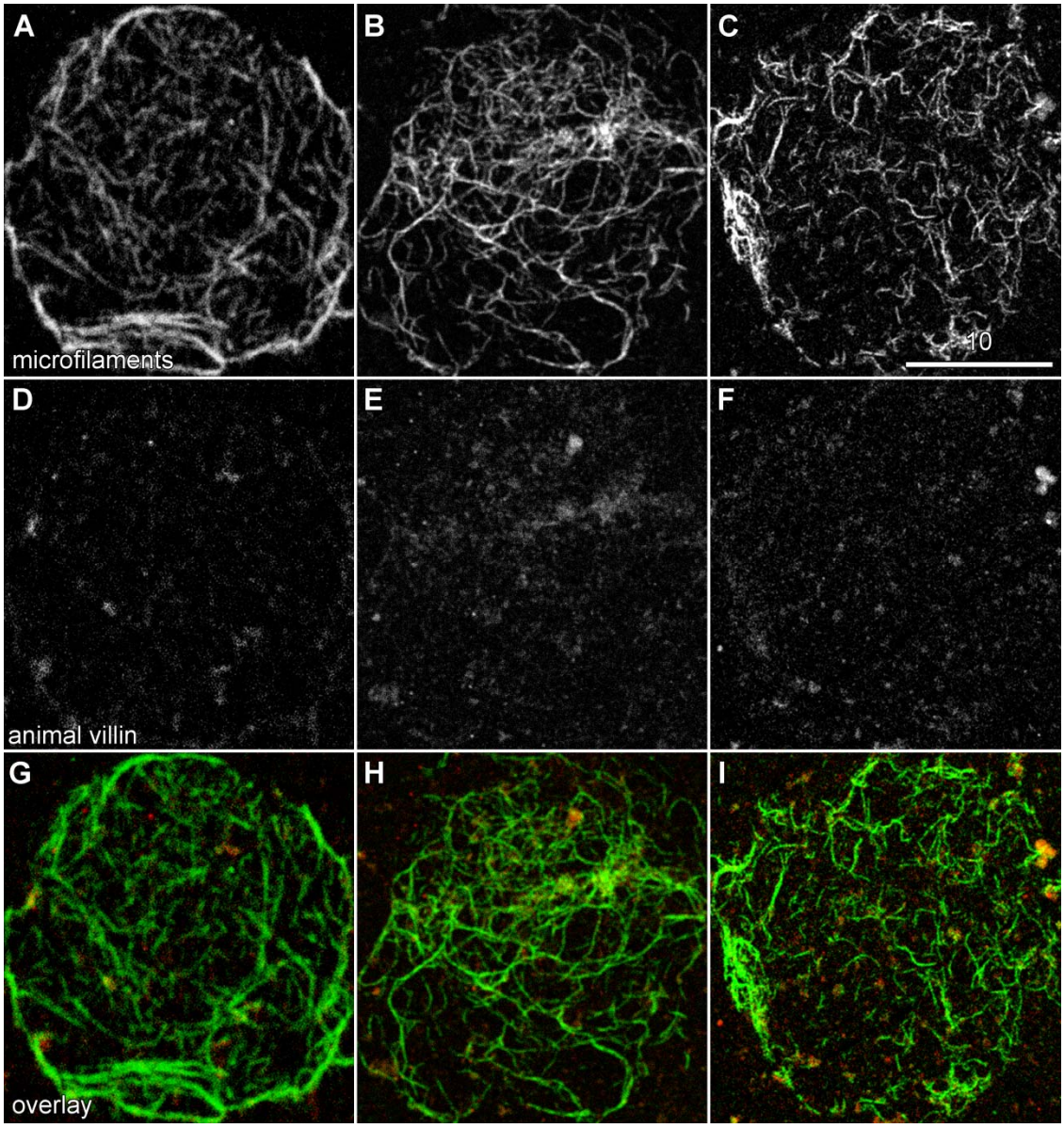

Figure 3.36. Immunolabelling of 3 membrane ghosts with polyclonal animal anti-villin. Scale bar in **C** = 10 μm.

A,B,C Monoclonal anti-actin labelling of microfilaments.

D,E,F Animal anti-villin. No labelling of ghosts was present.

G,H,I Overlay - actin microfilaments in green and animal villin labelling in red.

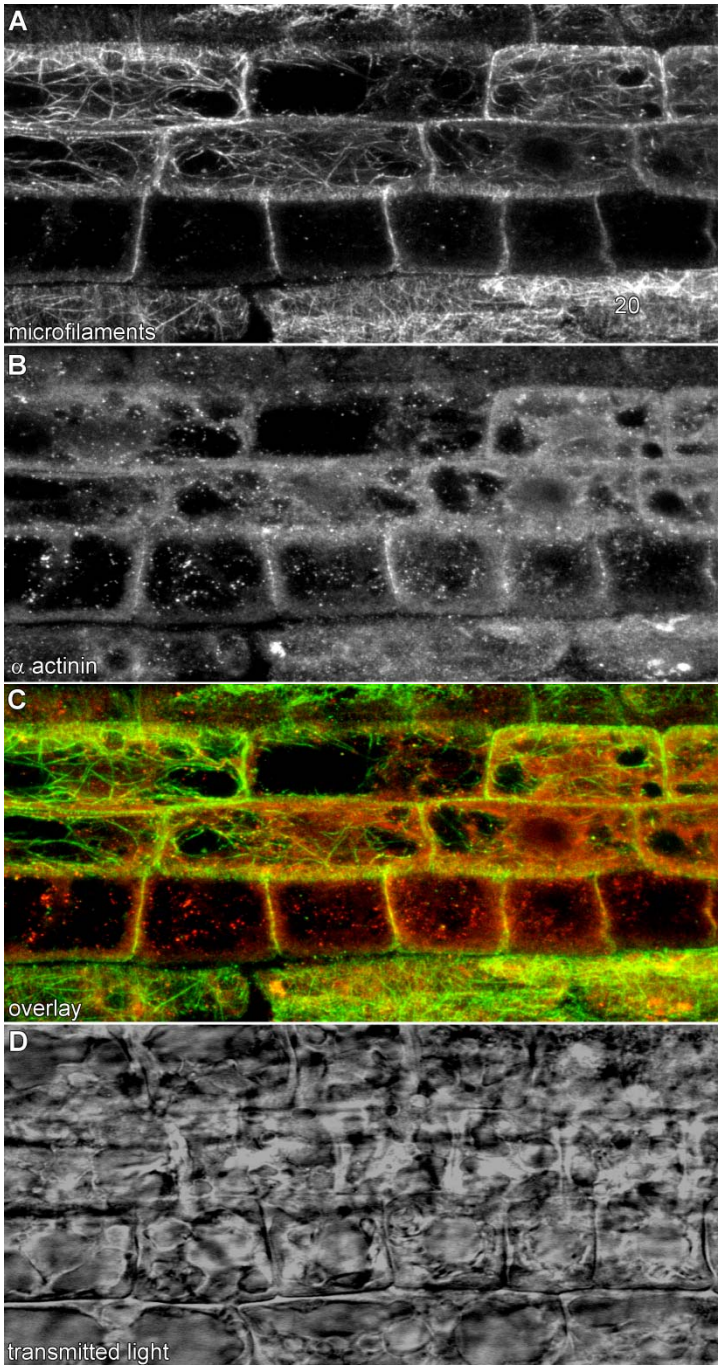

Figure 3.37. Immunolabelling of *Arabidopsis* root cells with polyclonal anti-α-actinin. Scale bar in **A** = 20 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-α-actinin - labelling was present as many small dotes scattered throughout the tissue. As this included the vacuoles of the elongating cells, this is likely artefactual.

C Overlay - actin microfilaments in green and α-actinin labelling in red.

D Transmitted light.

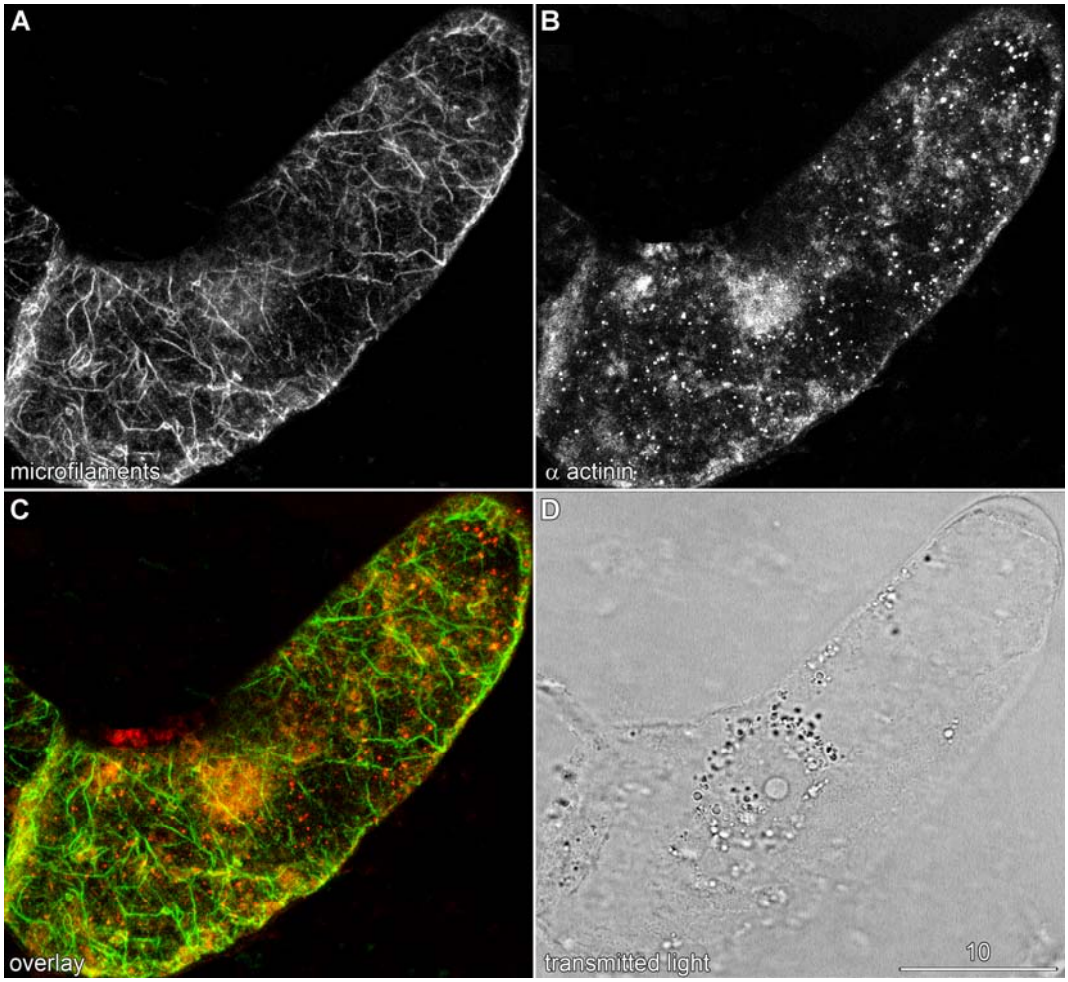

Figure 3.38. Immunolabelling of tobacco whole cells with polyclonal anti-α actinin. Scale bar in **D** = 10 μm

A Monoclonal anti-actin labelling of microfilaments.

B Anti-α-actinin; labelling was confined to speckles seen throughout the cells in a similar pattern to that seen in *Arabidopsis* roots.

C Overlay - actin microfilaments in green and α-actinin labelling in red.

D Transmitted light.

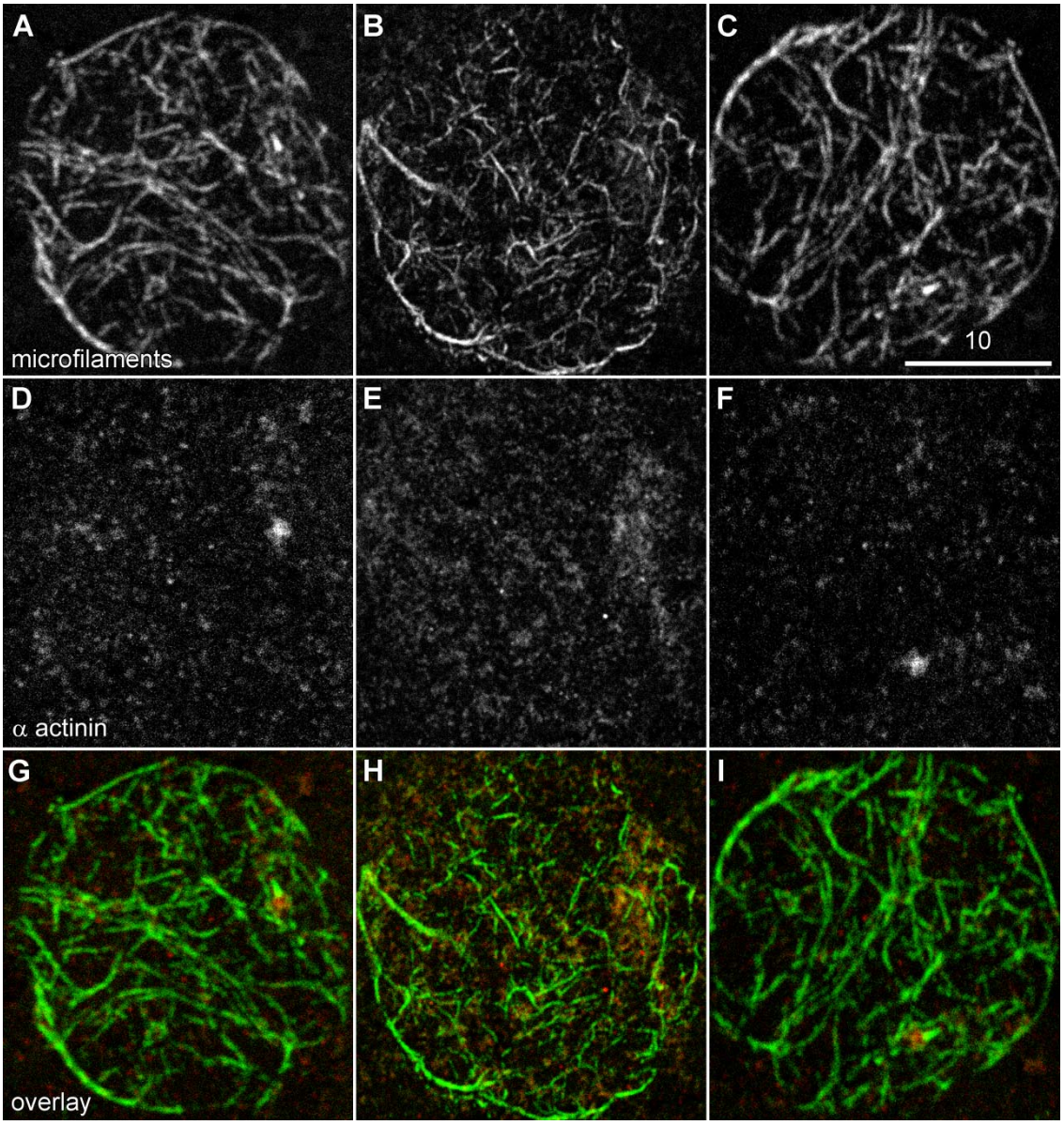

Figure 3.39. Immunolabelling of 3 membrane ghosts with polyclonal anti-α-actinin. Scale bar in **C** = 10 μm.

A,B,C Monoclonal anti-actin labelling of microfilaments.

D,E,F Anti-α-actinin. No labelling of ghosts was present.

G,H,I Overlay - actin microfilaments in green and α-actinin labelling in red.

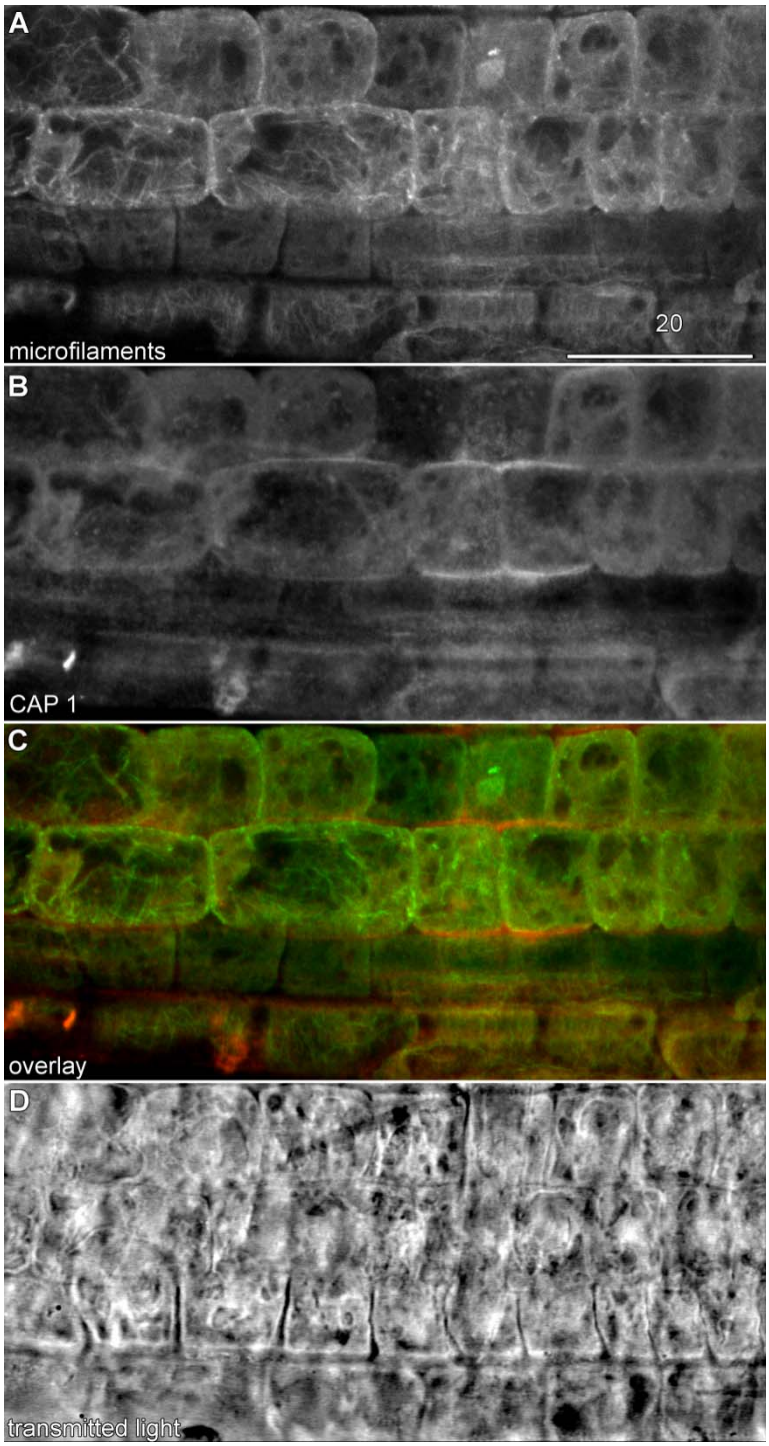

Figure 3.40. Immunolabelling of elongating *Arabidopsis* root cells with polyclonal anti-plant CAP. Scale bar in **A** = 20 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Labelling of with antibodies against CAP showed a general, cytoplasmic distribution.

C Overlay - actin microfilaments in green and CAP labelling in red.

D Transmitted light.

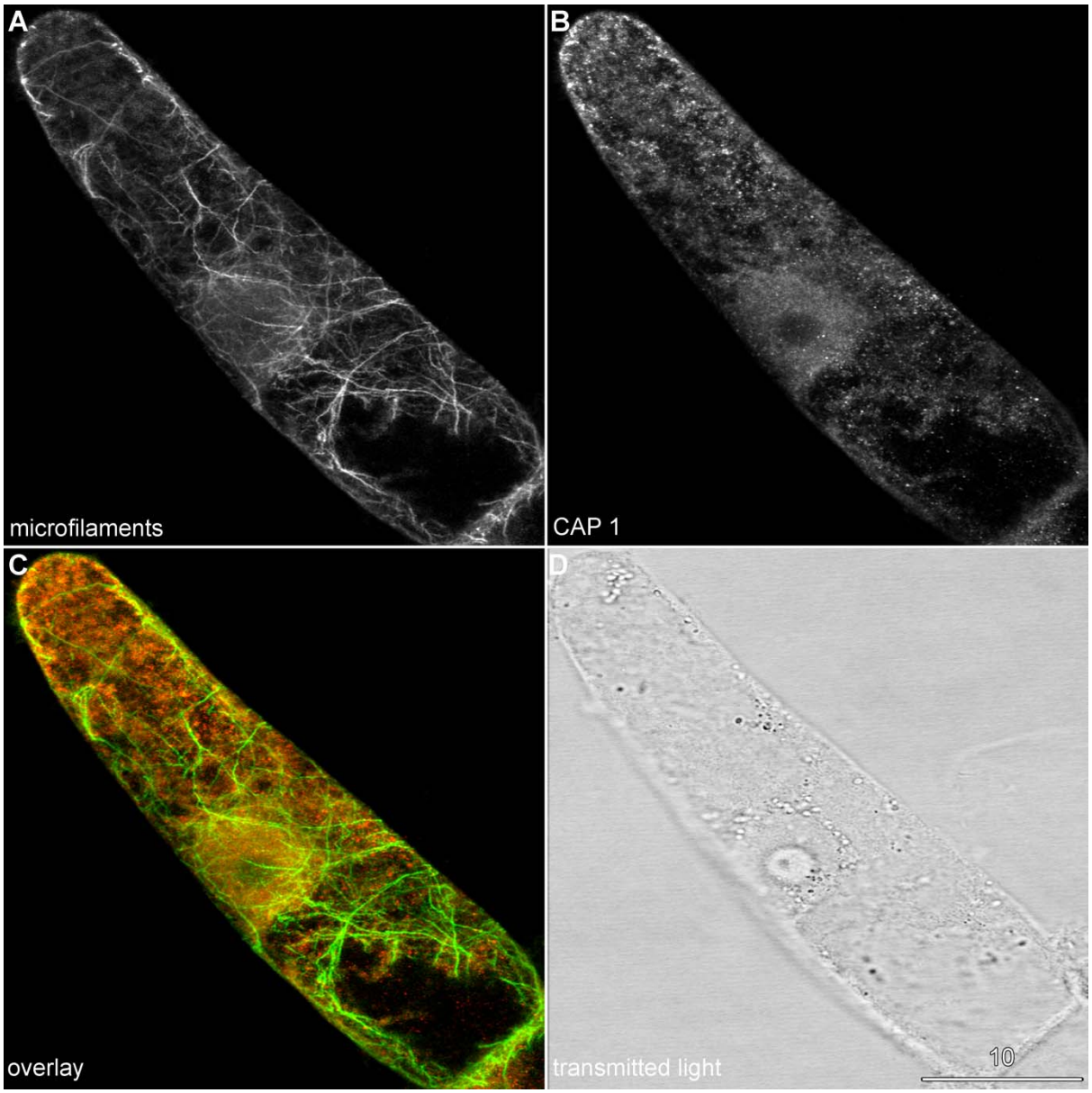

Figure 3.41. Immunolabelling of tobacco BY2 cells with polyclonal anti-CAP. Scale bar in **D** = 10 µm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-CAP. Little labelling was present.

C Overlay - actin microfilaments in green and CAP labelling in red.

D Transmitted light.

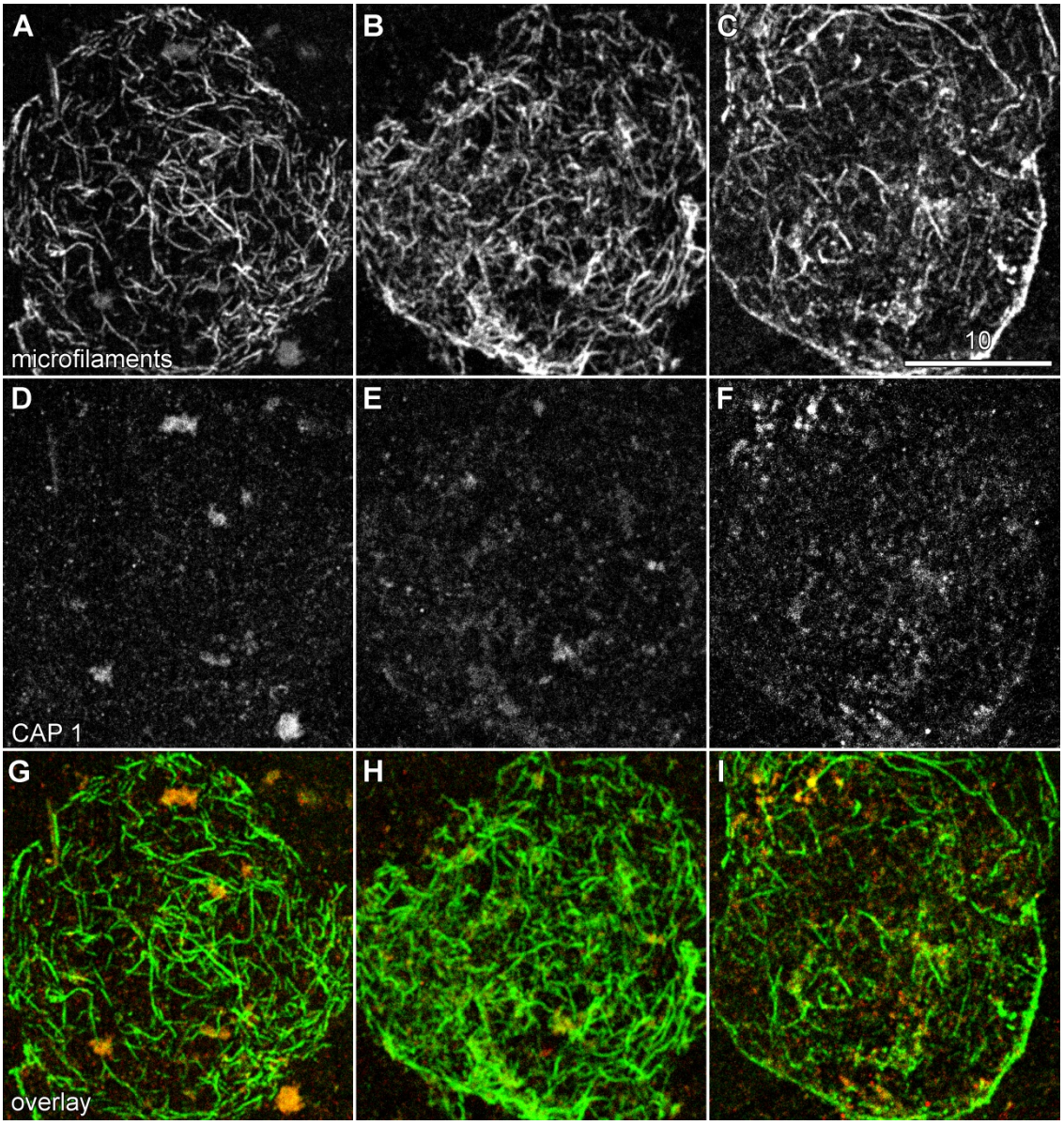

Figure 3.42. Immunolabelling of 3 membrane ghosts with polyclonal anti-CAP. Scale bar in **C** = 10 µm.

A,B,C Monoclonal anti-actin labelling of microfilaments.

D,E,F Anti-CAP. No labelling of ghosts was present.

G,H,I Overlay - actin microfilaments in green and CAP labelling in red.

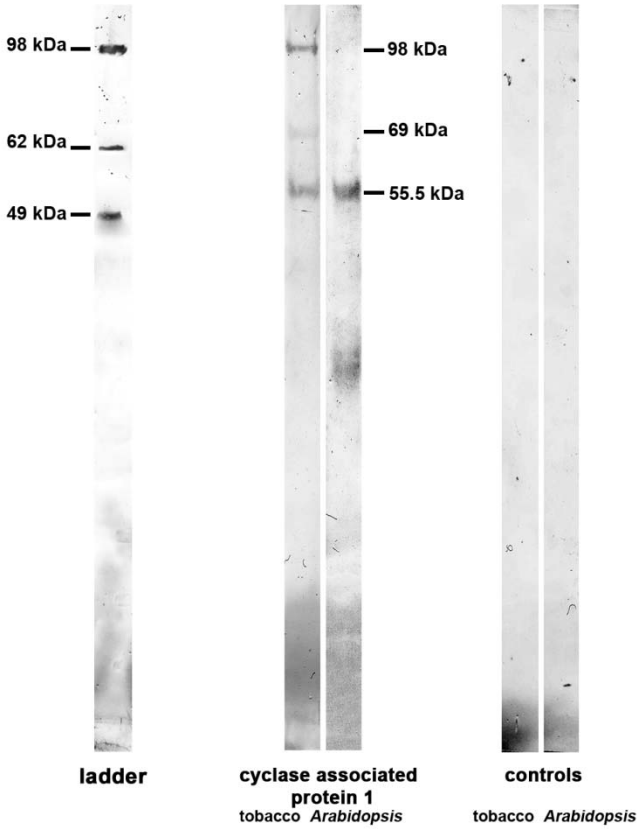

Figure 3.43. Western blotting analysis of cyclase associated protein 1 in *Arabidopsis* root cells and tobacco BY2 whole cells

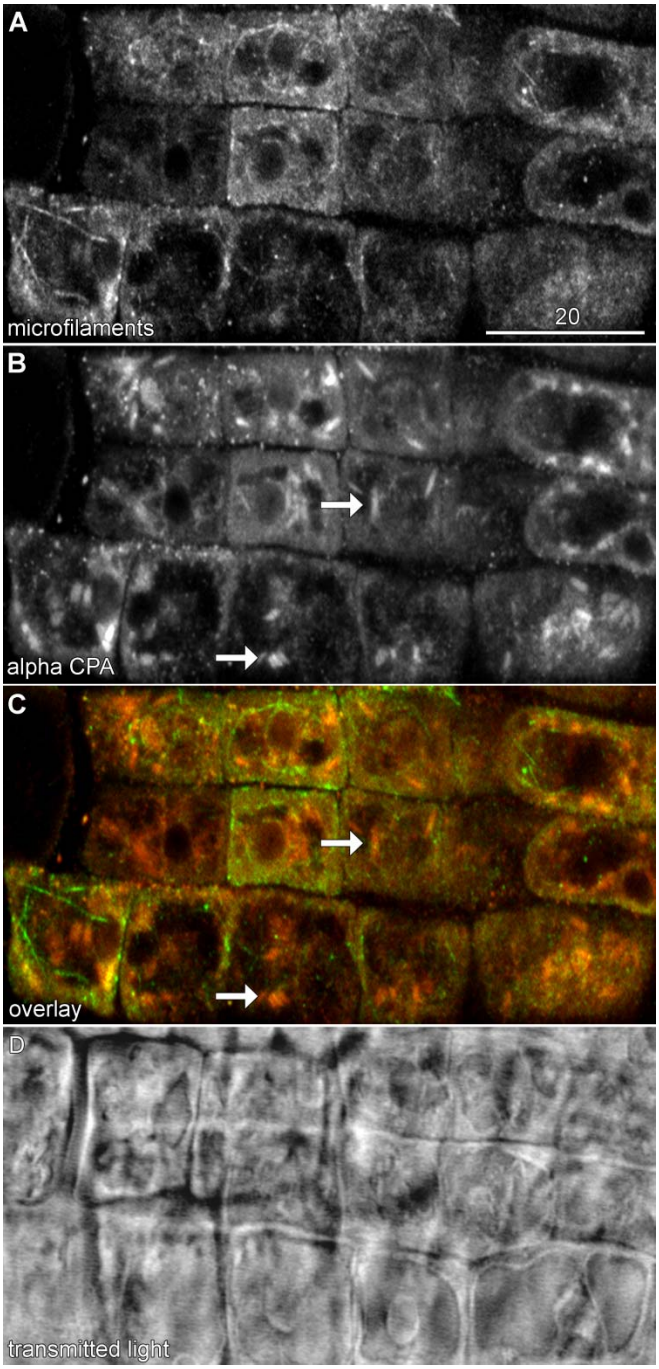

Figure 3.44. Immunolabelling of elongating *Arabidopsis* root cells with polyclonal anti-α CPA. Scale bar in **A** = 20 μm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-α CPA. While labelling was generally cytoplasmic, the antibody also labelled elongated organelle-like structures (arrows) consistent with the distended endoplasmic reticulum found in elongating root cells expressing ER-targeted GFP.

C Overlay - actin microfilaments in green and α-CPA labelling in red.

D Transmitted light.

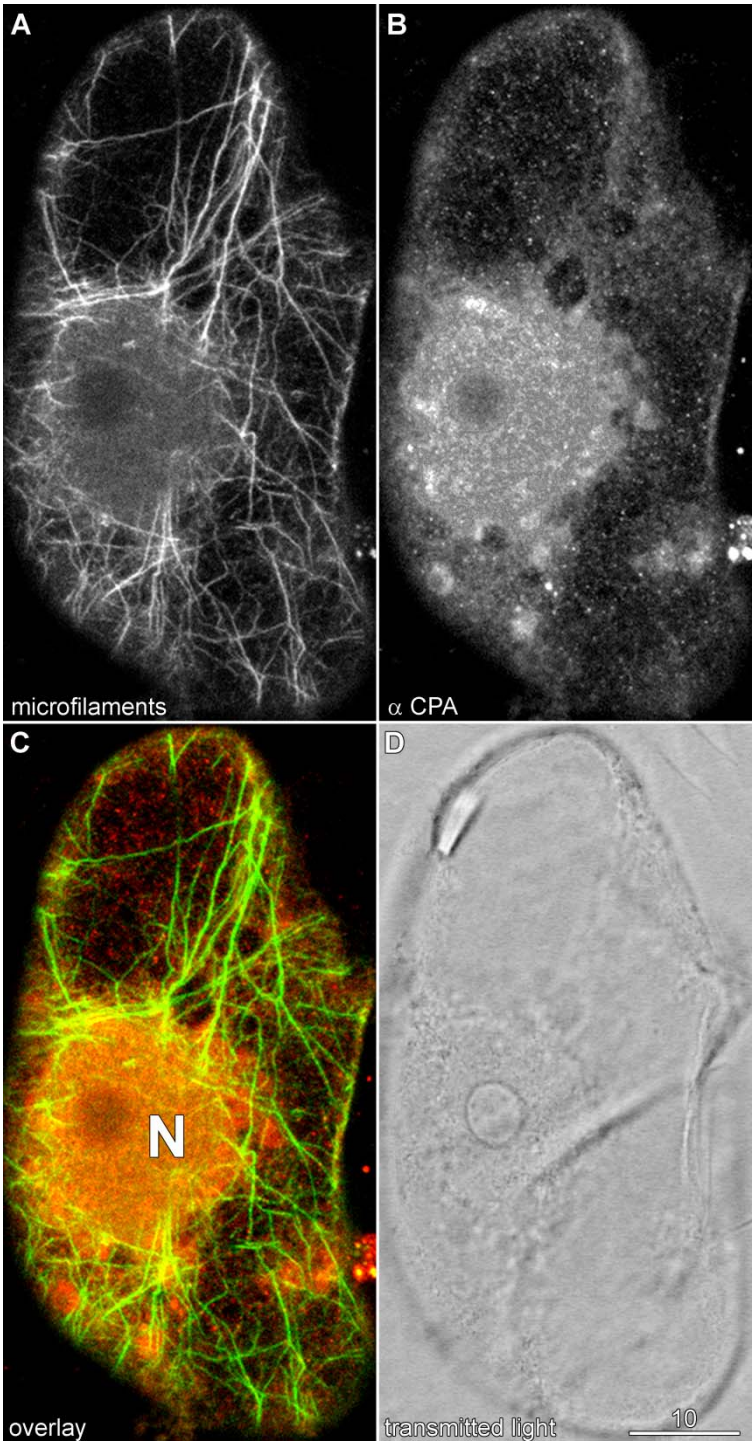

Figure 3.45. Immunolabelling of tobacco BY2 cells with polyclonal anti-α-capping protein. Scale bar in **D** = 10 μm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-α-capping protein. Labelling was present of membranous areas, notably around the nucleus (N) although no cytoskeletal labelling was present.

C Overlay - actin microfilaments in green and α-capping protein labelling in red.

D Transmitted light.

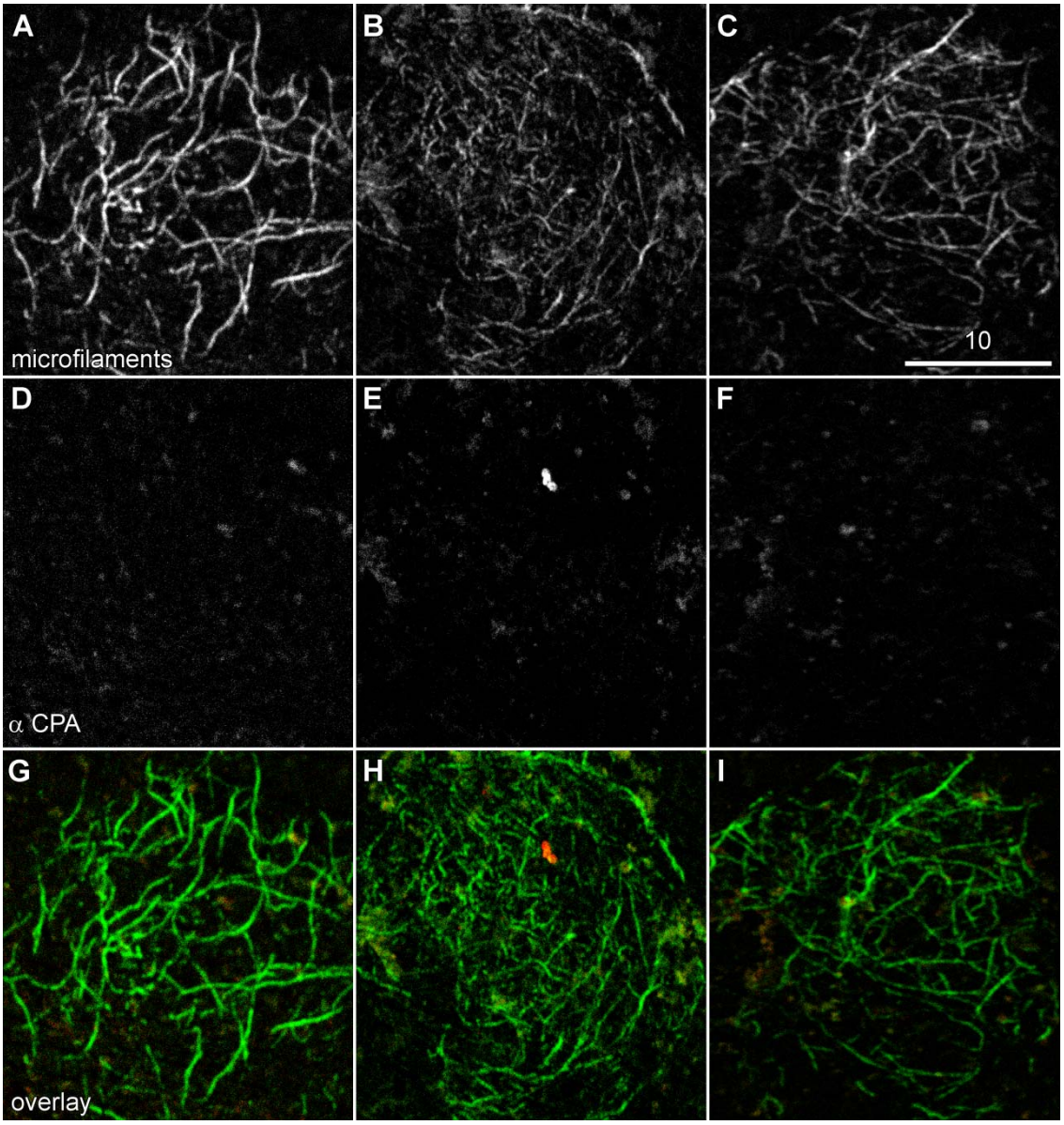

Figure 3.46.. Immunolabelling of 3 membrane ghosts with polyclonal anti-α capping protein. Scale bar in **C** = 10 μm.

A,B,C Monoclonal anti-actin labelling of microfilaments.

D,E,F Anti-α capping protein. No ghost labelling as present.

G,H,I Overlay - actin microfilaments in green and α capping protein labelling in red.

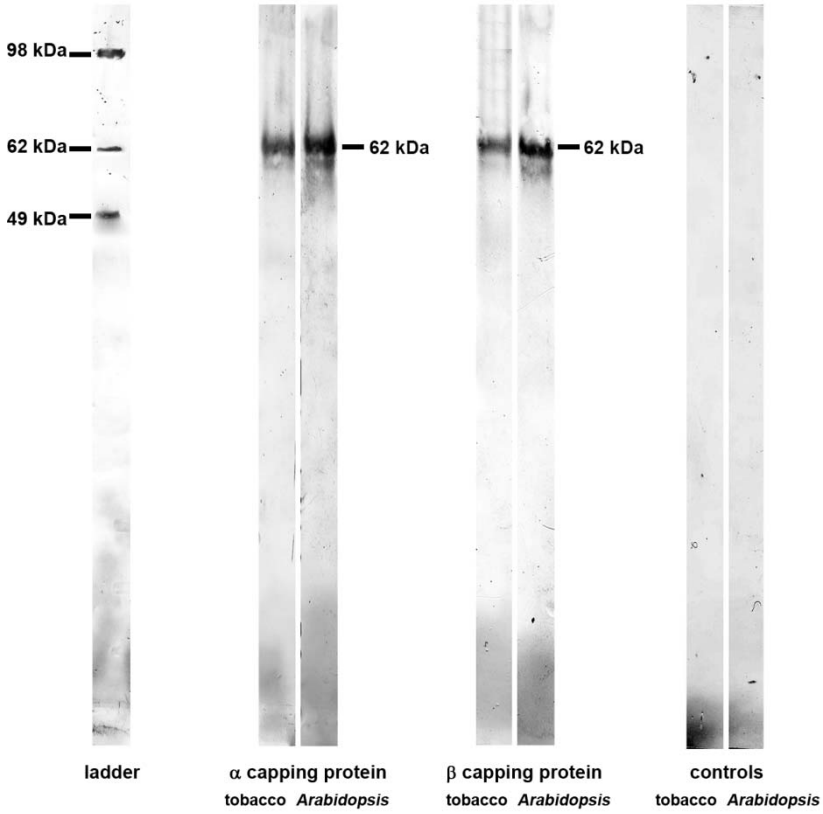

Figure 3.47. Western blotting analysis of α and β capping protein in *Arabidopsis* root cells and tobacco BY2 whole cells.

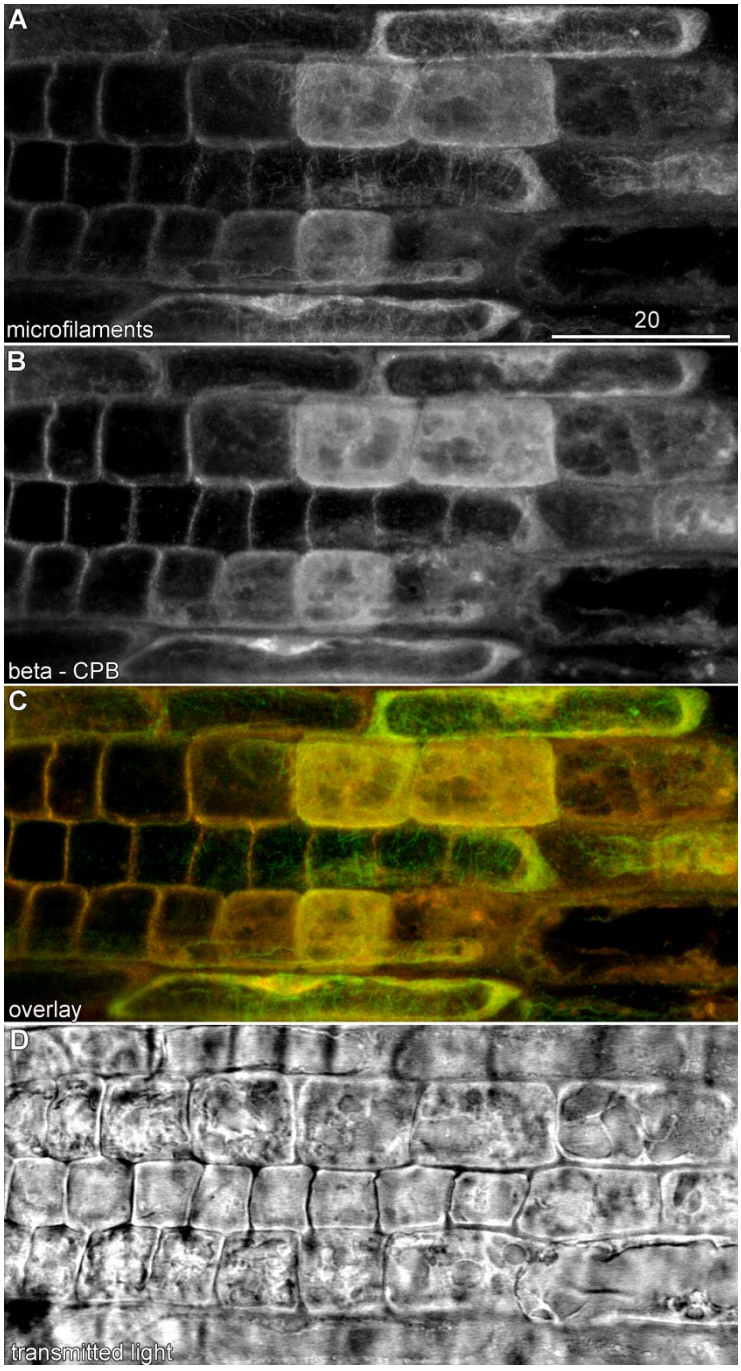

Figure 3.48. Immunolabelling of elongating *Arabidopsis* root cells with polyclonal anti-β CPB. Scale bar in **A** = 20 μm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-β CPB. Significantly, cells that labelled well with anti-actin also labelled strongly with anti-β CPB, although distinct co-localisation was not evident.

C Overlay - actin microfilaments in green and β-CPA labelling in red.

D Transmitted light

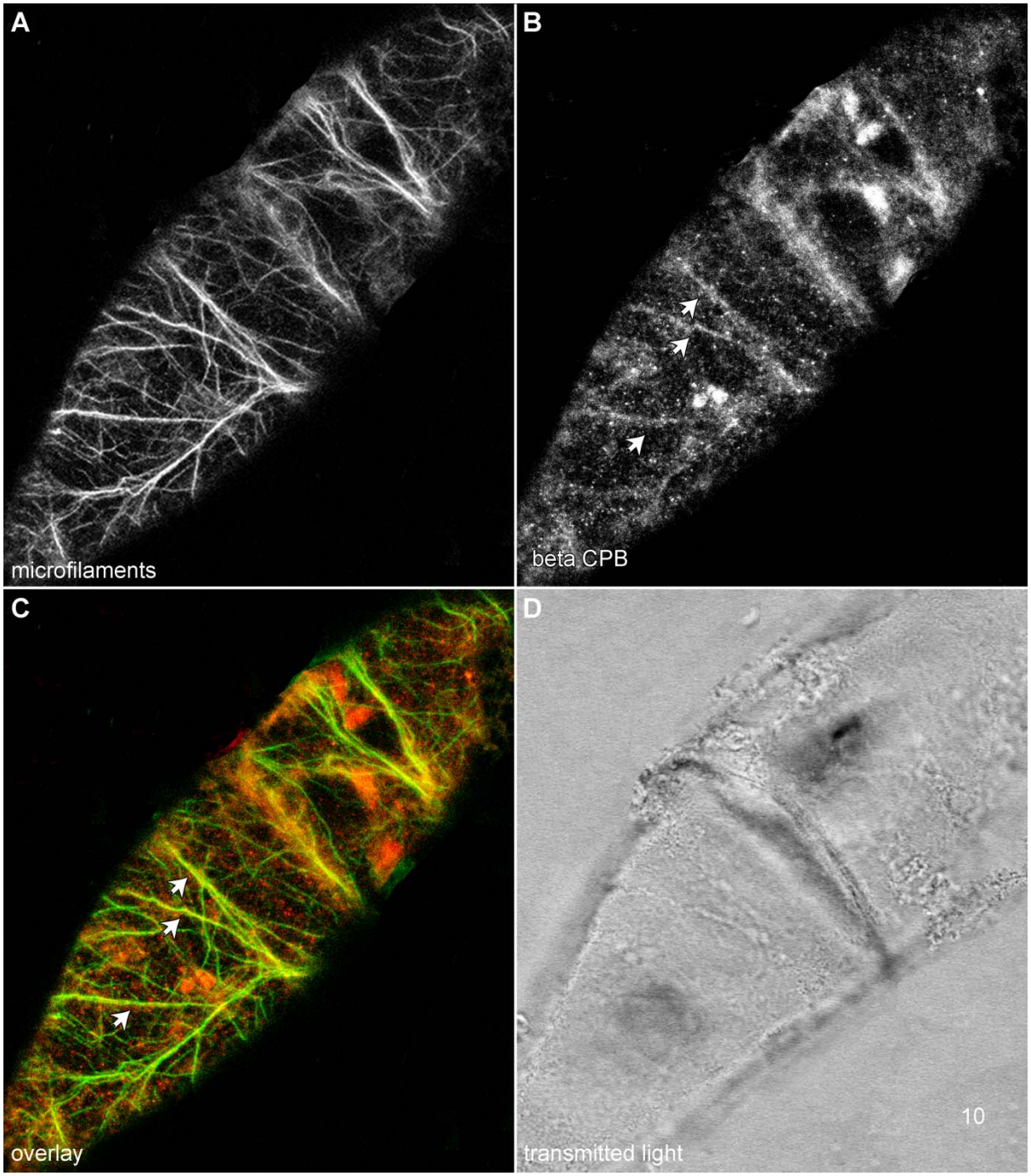

Figure 3.49. Immunolabelling of tobacco BY2 cells with polyclonal anti-β-capping protein demonstrates possible co-localisation with actin microfilaments. Scale bar in **D** = 10 μm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-β-capping protein. Intriguing bands and lines were present, including some that matched the thicker microfilament bundles (arrows).

C Overlay - actin microfilaments in green and β-capping protein labelling in red.

D Transmitted light.

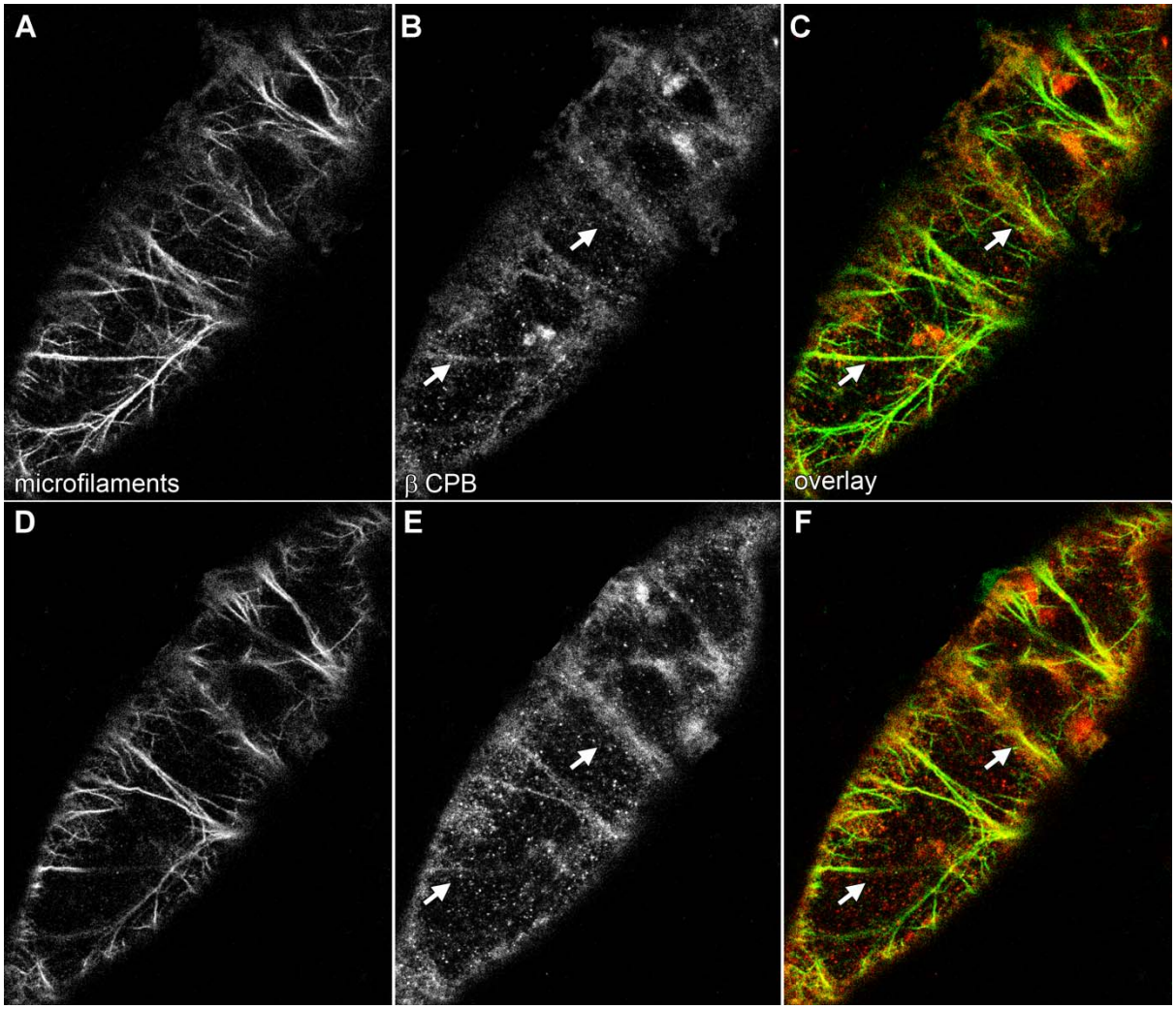

Figure 3.50. Two optical sections showing anti-β capping protein labelling in tobacco BY2 cells. The planes were separated by 1 μm. Scale bar in **C** = 10 μm.

A,D Monoclonal anti-actin labelling of microfilaments.

B,E Anti-β capping protein. Several examples of lines are present within this image that co-localise with the thicker microfilament bundles present in panel **A**.

C,F Overlay - actin microfilaments in green and β capping protein labelling in red.

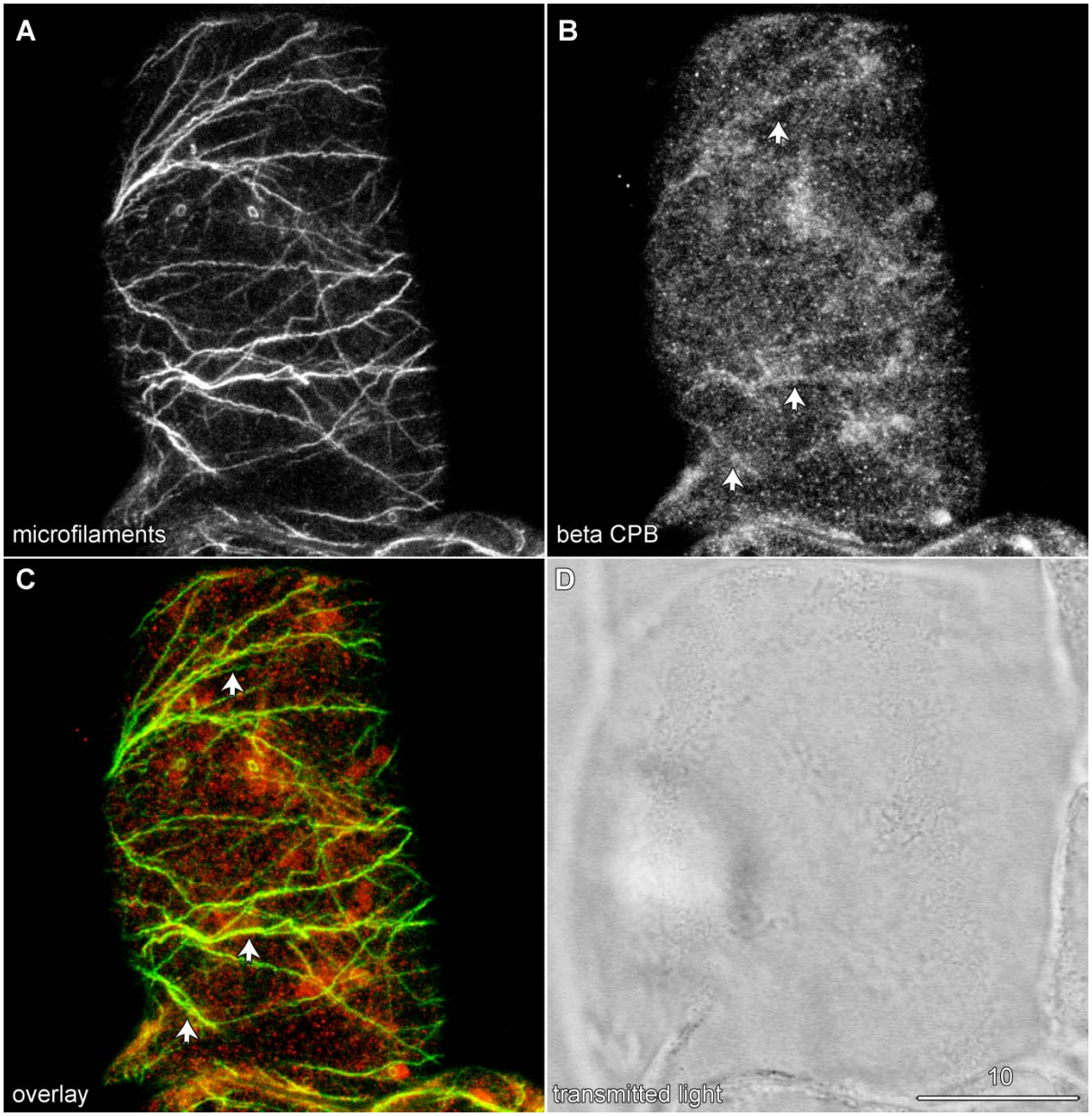

Figure 3.51. A second example of co-localisation between β-capping protein and actin microfilaments in tobacco BY2 cells. Scale bar in **D** = 10 μm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-β-capping protein. Several examples of co-localisation with microfilaments are highlighted (arrows).

C Overlay - actin microfilaments in green and β-capping protein labelling in red.

D Transmitted light.

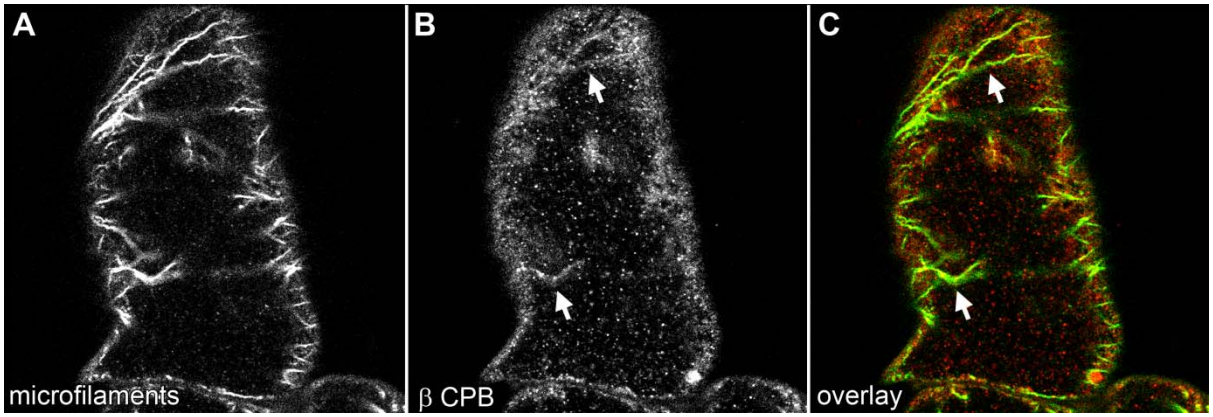

Figure 3.52. A single optical section showing the co-localisation of anti-β capping protein labelling with actin microfilaments in tobacco BY2 cells. Scale bar in **C** = 10 μm.

A Monoclonal anti-actin labelling of microfilaments.

B Anti-β capping protein. Examples of co-localisations are indicated with arrows.

C Overlay - actin microfilaments in green and β capping protein labelling in red.

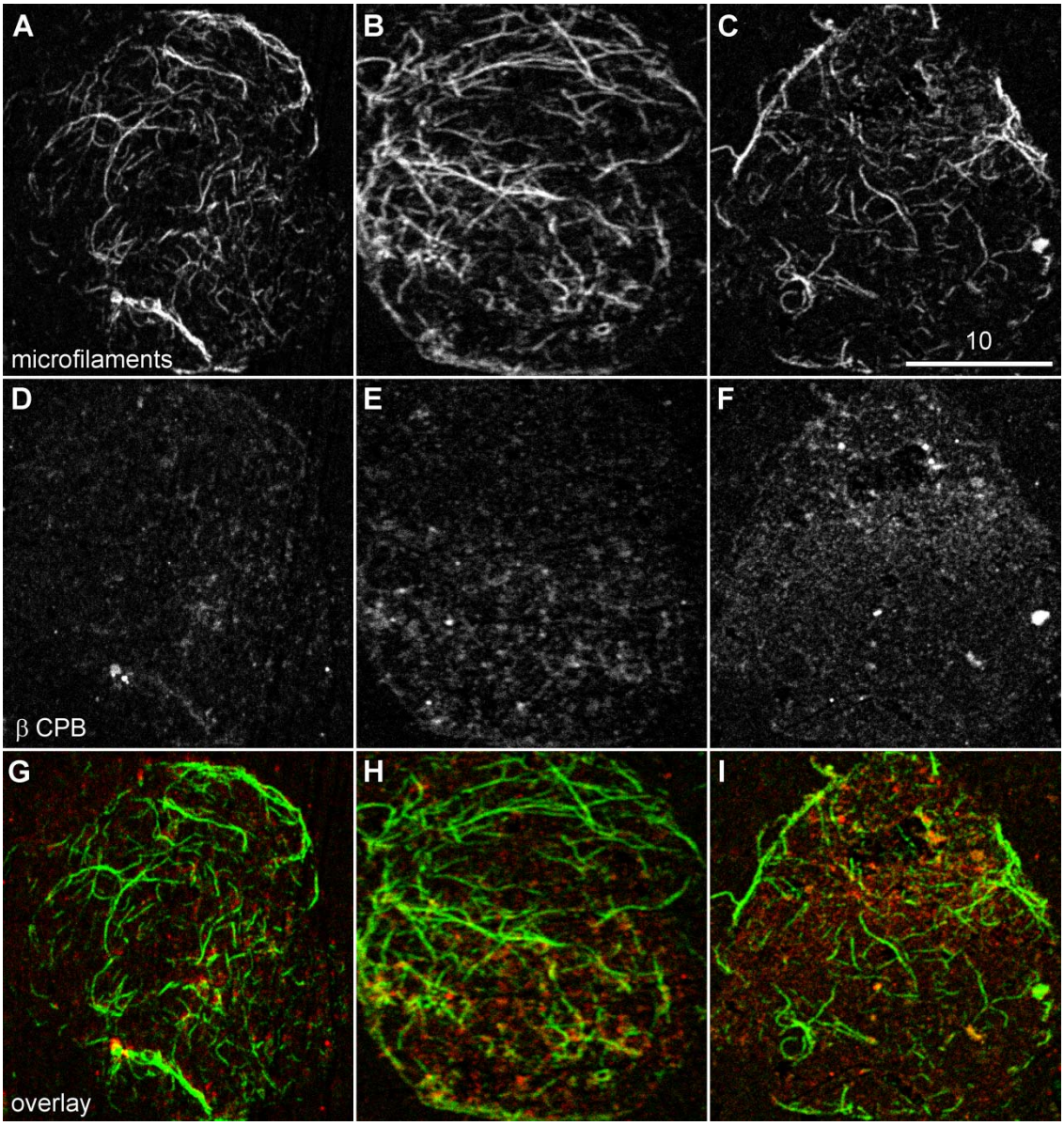

Figure 3.53.. Immunolabelling of 3 ghosts with polyclonal anti-β capping protein. Scale bar in **C** = 10 μm.

A,B,C Monoclonal anti-actin labelling of microfilaments.

D,E,F Anti-β capping protein. No ghost labelling was present.

G,H,I Overlay - actin microfilaments in green and α capping protein labelling in red.

Table 3.1. Summary of immunolabelling and immunofluorescence results

antigen	*Arabidopsis roots*		Tobacco BY2 cells		
	immunofluorescence	western blots	whole cells - immunofluorescence	ghosts - immunofluorescence	western blots
Monoclonal anti tubulin clone B512	✓	✓	✓	✓	✓
Polyclonal anti – actin	✓	✓	✓	✓	✓
Monoclonal C4 anti – actin	✓	✓	✓	✓	✓
Polyclonal anti – tropomyosin	✓	✓	×	×	✓
Polyclonal anti – fimbrin	?	✓	?	×	✓
Polyclonal anti – villin from plant	?	✓	?	×	✓
Polyclonal anti – villin from animal	×	✓	×	×	✓
Polyclonal anti – α actinin	×	✓	×	×	✓
Polyclonal anti – caldesmon	✓	×	?	×	×
Polyclonal anti – cyclase association protein 1	×	✓	×	×	✓
Polyclonal anti α capping protein	✓	✓	?	×	✓
Polyclonal anti β capping protein	?	✓	✓	×	✓

CHAPTER 4

Discussion

Actin-binding proteins are important in the proper functioning of a plant cell as they determine the configuration and dynamic of actin microfilaments and thus play a crucial role in cellular functionality. While actin monomer sequences and microfilament structure are highly conserved across eukaryotes, actin-binding proteins are less well conserved, and the number and diversity of actin-binding proteins varies from organism to organism . This has made identifying and characterising these proteins difficult (Hussey et al. 2002).

Several proteins with homologies to known actin-binding proteins have been identified in the *Arabidopsis* genome. These include proteins like fimbrin (McCurdy and Kim 1998; Su et al. 2012; Wu et al. 2010), villin (Huang et al. 2005; Klahre et al. 2000; Nakayasu et al. 1998; Yokota et al. 2003), capping protein (Huang et al. 2003; Huang et al. 2006), myosin (Shimmen et al. 2000), profilin and ADF/cofilin (Staiger et al. 1997). The effects of these actin-binding proteins on actin dynamics has been shown by indirect and biochemical methods. However, studies that visualise the localisation of these proteins with the actin cytoskeleton have been lacking in plants, in contrast to the well documented literature on the localisation of actin-binding proteins in animal and fungal cells.

The aim of this project was to investigate the co-localisation of the actin-binding proteins with actin microfilaments. *Arabidopsis* and tobacco BY2 systems were chosen as ideal experimenting systems as they have been used in previous experiments to study the cytoskeleton and reliable data available for these systems was used in this project. Membrane ghosts derived from tobacco BY2 cells were also used for this was thought to be a way of reducing the background labelling of the cytoplasm which can be problematic when observing actin-binding proteins.

Labelling of microtubules with anti-tubulin and actin microfilaments with polyclonal anti-actin was used as a positive control for *Arabidopsis* roots, tobacco BY2 cells and membrane ghosts throughout the thesis. Cytoskeletal labelling patterns seen in the cells and ghosts were similar to published data (Collings et al. 1998; Collings et al. 1999; Collings and Wasteneys 2005; Faulkner et al. 2009; Wasteneys and Collings 2007). Control experiments proved that the secondary antibodies were specific to the primary antibodies and that the confocal microscope settings used for imaging could be optimised so that there was no overlapping of fluorescence signals. This involved using sequential imaging so that the two secondary antibodies used were not excited at the same time, by using spectrally well-separated second antibodies (fluorescein and Alexa-594 were found to be best), and by labelling the actin-binding protein in green and the actin cytoskeleton in red or far red so that the actin image did not bleed through into the actin binding protein channel. Further, because the whole cells of *Arabidopsis* and tobacco contain the cytoplasm, ER, nuclei and other organelles that might generate non-specific binding of antibodies, membrane ghosts produced from tobacco BY2 cells were

developed as tool to eliminate the cytoplasm and other organelles. These methods were all developed so that an optimal localisation of actin-binding proteins to actin cytoskeleton might be made.

4.1. Problems with Immunoblotting

The identification of putative actin-binding proteins by immunofluorescence requires concurrent evidence for the presence of immunoreactive proteins by immunoblotting. Unfortunately, numerous problems were detected in the immunoblotting experiments, and some results appear to be difficult to interpret or contradictory to previously published data.

Although the monoclonal α-tubulin antibody detected a 55 kDa protein consistent with α-tubulin in both tobacco and *Arabidopsis* cells, problems were experienced with the same proteins solutions with the actin antibodies. Actin is usually recognised as a single specific band at 42 kDa in western blotting analyses. However, no band was detected with the monoclonal anti-actin antibody although this has been shown to work on western blots of plant tissue (Blackman and Overall 1998)and multiple bands (64, 56 and 47 kDa) were seen with polyclonal anti-actin again inconsistent with previous results which demonstrate a single 42 kDa band (Gibbon et al. 1999). That the problem likely lies with either protein extraction and / or the western blotting technique is demonstrated by the fact that immunolabelling experiments for both monoclonal and polyclonal anti-actin were specific to actin microfilaments, and that the patterns seen in the cells and ghosts were similar those in literature.

Numerous of the actin-binding protein antibodies also gave anomalous molecular weights when they detected bands in the *Arabidopsis* and tobacco extract. Again, the reasons for this remain unknown.

4.2. Immunolabelling with Antibodies against Actin-Binding Proteins

4.2.1. Tropomyosin

The labelling pattern of tropomyosin in root cells was tantalisingly different from the labelling pattern of actin. Actin labelled the phragmoplast, the periphery of the dividing cells and non-dividing root cells (Collings and Wasteneys 2005; Faulkner et al. 2009; Voigt et al. 2005). In contrast, tropomyosin localisation was distinct in the cell plates of dividing root cells and also was abundant in the cytoplasm of the dividing cells. The distribution of tropomyosin in the cytoplasm was reduced as the cells neared completion of cell division. These patterns of labelling have not been seen in previously published results (Faulkner et al. 2009). The results suggest that the tropomyosin-related protein may play an integral part during cytokinesis in *Arabidopsis* root cells. Western blotting results of *Arabidopsis* roots recognised bands at 71 kDa and 60 kDa which are quite different from the molecular weights of the sequence annotated as “tropomyosin-like” proteins in the *Arabidopsis* Information Resource (TAIR) website (http://www.*Arabidopsis*.org/) and

(http://www.ncbi.nlm.nih.gov/). The annotated sequences are computational and they have not been identified as functional tropomyosin (Faulkner et al. 2009). Also, the molecular weights of the bands identified in this study in *Arabidopsis* and tobacco are markedly different to those identified by Faulkner and colleagues in *Arabidopsis* (42.5 kDa), although similar to the molecular weights identified in the alga *Chara* (Faulkner et al. 2009).

4.2.2. Caldesmon and α-actinin

Polyclonal and monoclonal anti-caldesmon antibodies raised against the N-terminal fragment of chicken gizzard identified a 107 kDa caldesmon-like protein and the protein was found to interact with actin in a Ca^{2+} -dependent manner in *Ornithogalum virens* pollen tubes (Wu et al. 2010). In our experiments, polyclonal anti-caldesmon raised against the C-terminal of the human caldesmon labelled the nucleus of the vasculature in the elongation zones of root cells which likely represents non-specific binding. No other labelling was detected, and the western blot results were negative.

Experiments also identified a protein with a molecular weight of ~ 58 kDa in the *Arabidopsis* root extracts using an antibody against chicken gizzard α-actinin, although no bands were seen in the tobacco BY2 cell extracts. This is the first experimental evidence for presence of α-actinin like protein in *Arabidopsis*. However, an 80 kDa α-actinin like protein was isolated from *Lillium davidii* and was found to associate with Golgi as punctuate dots in the cytoplasm in the pollen tubes (Li and Yen 2001). Antibodies against α-actinin did not co-localisation to the actin microfilaments but were seen dispersed throughout the cytoplasm and vacuole of the cells.

4.2.3. Capping protein

Antibodies against the α-subunit of capping protein labelled structures in the *Arabidopsis* root cells that was not related to the actin cytoskeleton. It is believed that the antibody may be associating with the endoplasmic reticulum. Similar structures, resembling those seen in the α-CPA labelling, are observed with GFP constructs targeted to the ER of *Arabidopsis* and was found to be most abundant in the root tissue (Haseloff et al. 1997; Gunning 1998; Hawes et al. 2001). These structures have not been observed in other GFP targeted systems such as tobacco cells (Boevink et al. 1999). This could explain why there was no labelling in the tobacco BY2 whole cells or ghosts. The labelling pattern seen with this antibody is unusual because capping protein specifically binds to the barbed end of the actin filament and is present mainly in the cytoplasm of animal cells (Amatruda et al. 1990; Huang et al. 2003; Wear et al. 2003).

The antibodies against β-subunit of capping protein did not localise to the actin cytoskeleton or to the ER in the *Arabidopsis* roots. However, β-CPB co-localised to the thick actin bundles in the tobacco BY2 whole cells and punctuate spots was seen in the cytoplasm of the cells. The fluorescent imaging

studies previously conducted by Huang and colleagues to visualise the binding site of β-CPB was performed on isolated single filaments alone, which does not explain where the β-CPB localises in the whole cells (Huang et al. 2003). Immunolocalisation studies on ghosts did not recognise distinguishable localisation of the proteins but varying levels of punctuate labelling was seen for the different proteins tested. Usually, fine actin microfilaments with some thicker bundles were retained on the ghosts made from tobacco BY2 cells (Collings et al. 1998; Collings et al. 1999) and cultured *Zinnia* cells (Kobayashi 1996) and similar results were seen in the present study. The labelling pattern in tobacco cells with β-CPB indicates that the proteins associated with the thicker actin bundles. Since the finer actin filaments were mainly retained in the ghost cells, the antibody against β-CPB could not localise to actin microfilaments on ghosts. But if the antibodies were able to localise to barbed end of single filaments they should typically localise to the actin cytoskeleton in the ghost system. Typically the alpha subunit of capping protein should have a labelling pattern similar to that seen in the beta subunit labelling but the antibody against α-CPA recognises the ER.

Comparison of sequence homologies between *Arabidopsis* α-CPA (AtCPA) and β-CPA(AtCPB) subunits and their homologues in vertebrates and yeast indicated that the β subunit had a higher conservation (40 – 50%) than the α subunit (25 – 30%). It was also found that the C-terminal regions of both subunits involved in binding to F-actin were especially poorly conserved when compared to the vertebrate homolgues. The molecular weights of α and β subunits was found to be 39 kDa and 31 kDa respectively (Huang et al. 2003), significantly different to the band sizes detected in this set of experiments for *Arabidopsis* and tobacco. The molecular weights of α and β subunits were inconsistent with data found in previously published results. Although the western blotting results prove that the antibodies were present in *Arabidopsis* and tobacco systems, we cannot conclusively say that the antibodies were specific to the proteins being studied.

4.2.4. Fimbrin, Villin and CAP1

Fimbrin antibodies identified bands of molecular weights ~ 73 kDa (*Arabidopsis* roots) and ~ 64 kDa (tobacco BY2 cells) in the western blotting experiments, roughly consistent with previous estimates of these well-characterised plant microfilament bundling proteins. Similarly, cyclase associated protein 1 (CAP1) recognised bands at ~ 55.5 kDa, similar to both the calculated molecular weights from annotated sequences and experimental data previously published (Kovar et al. 2000b). However, no co-localisation was present with the actin cytoskeleton in the *Arabidopsis* roots, tobacco BY2 cells or ghosts.

Villin was recognised as multiple bands in the tobacco samples using antibodies against plant and animal villin. In the root extracts, a single band for villin was detected by plant anti-villin but animal anti-villin failed to detect any proteins. Villin has a large molecular weight ranging between 102 kDa

to 135 kDa (Huang et al. 2005; Khurana et al. 2010; Yokota et al. 2005; Yokota et al. 2003).The proteins could have undergone proteolytic degradation during extraction procedures which could explain the detection of multiple bands during western blotting.

4.3. Conclusions and Further Experiments

Even in the era of GFP fusion proteins and live cell imaging, immunolabelling studies remain important for understanding the cellular functions of proteins. Inability of the antibodies to stain tissue in cells does not necessarily mean that the antigens are absent in the cells. Often times the antibodies recognise denatured proteins and fail to interact with proteins in their native form. This results in positive staining of protein bands on western blots that are denatured by SDS-PAGE gel electrophoresis (Brown et al. 1996; D'Amico et al. 2009). There are several aspects within an experimental setup, like the type of fixatives used, pH of the solutions, and presence of calcium ions affect the outcome of results. The actin stabilisation buffer containing pipes, EGTA and $MgCl_2$ was used in immunolabelling tobacco BY2 whole cells, protoplasts and ghosts to obtain stable actin microfilaments and microtubules. When the EGTA in the buffer was replaced with $CaCl_2$ microtubules were disrupted which resulted in ghosts containing only the actin cytoskeleton. Also, actin microfilaments were found to be more stable at acidic pH whereas the microtubules were found to be more stable at alkaline pH (Collings et al. 1998). Aldehydes like formaldehyde and glutaraldehyde are the most common chemical fixatives used in experiments. Formaldehyde is capable of cross-linking reaction between amino, imino, amido, guanidyl and hydroxyl groups and aromatic rings of proteins (Pearse 1980; Yamashita 2007). The effect of formaldehyde on cross-linking was demonstrated by SDS-PAGE experiments (Hopwood et al. 1988; Rait et al. 2004; Yamashita and Okada 2005). These alterations lead to changes in the three-dimensional structure of proteins, thus affecting the tertiary and quaternary structures of protein, whereas the primary and secondary structures remain unaffected (Dill and Shortle 1991; Mason and O'Leary 1991; Werner et al. 1996). The two functional groups of glutaraldehyde cause the formation of a stable and irreversible crosslinks (Eltoum et al. 2001). Glutaraldehyde affects the alpha helix structure of the proteins because of its strong fixing ability (Yamashita 2007). Because of these alterations, the epitopes of a protein become “masked” which makes them inaccessible to the antibodies. Essentially this does not mean that the epitopes are critically altered. Antigen retrieval techniques could be a useful tool to significantly increase the sensitivity for detection of epitopes for better morphological detail and more

precise antigen localisation (D'Amico et al. 2009; Iwamura et al. 1994; Sheriffs et al. 2001; Van den Berg et al. 1993)(D'Amico et al. 2009; Iwamura et al. 1994; Sheriffs et al. 2001; Van den Berg et al. 1993). This property can be an important tool to achieve better visualisation of the interaction between actin microfilaments and actin-binding proteins.

4.3 Conclusions and Further Experiments

Even in the era of GFP fusion proteins and live cell imaging, immunolabelling studies remain important for understanding the cellular functions of proteins. The experiments described in this thesis were aimed at determining where in plant cells specific actin-binding proteins are present, with the information coming from such studies critical in understanding how these proteins work in controlling the actin cytoskeleton.

Although the membrane ghosts system was thought to be a way of detecting weak signals for localisation patterns of actin-binding proteins, because of the ability to remove interference from cytoplasmic labelling, no conclusive colocalisation was observed with the range of antibodies used. while this is not necessarily surprising for the antibodies raised against animal actin-binding proteins, given the levels of conservation between these proteins and their plant homologues (where known), the lack of specific labelling patterns with the antibodies raised against known plant actin-binding proteins is more difficult to explain, especially as these antibodies are generally known to work on western blots.

Several possibilities exist to explain this puzzling difference. The inability of the antibodies to label cells does not necessarily mean that the antigens are absent from the cells. Antibodies typically recognise denatured proteins because they are screened against denatured proteins by western blotting during the antibody generation process, but may fail to interact with proteins that are in their native or chemically-fixed form. This results in positive staining of protein bands on western blots that are denatured by SDS-PAGE gel electrophoresis but a complete lack of labelling by immunofluorescence (Brown et al. 1996; D'Amico et al. 2009). It is possible, through certain specific steps, to modify the chemically-fixed antigens within tissue to make them more immunoreactive. These procedures, known as antigen recovery, may use a combination of heat treatments and high and low pH washes, or alternatively treatments with SDS or chaotropic agents such as urea (D'Amico et al. 2009). Initial attempts made with antigen retrieval, in order label of actin-binding proteins in whole cells and ghosts, failed because the retrieval steps eliminated the antigenicity of the actin such that the actin mcirofilaments were not recognised by the C4 monoclonal anti-actin. A careful balance of antigen retrieval and antigen stabilisation would, therefore, be necessary for these experiments to succeed.

Several aspects of the immunolabelling procedure might also be modified to improve the immunoreactivity and / or retention of actin-binding proteins in whole cells and ghosts. These include varying the fixatives used, pH of the solutions, and the presence of calcium ions. The cytoskeleton stabilisation buffer containing Pipes, EGTA and $MgCl_2$ used in immunolabelling *Arabidopsis* roots, tobacco BY2 cells and membrane ghosts gave good labelling of actin microfilaments and microtubules but might not be ideal for the retention of actin-binding proteins. Modifications to this buffer are possible: when the EGTA in the buffer was replaced with $CaCl_2$ microtubules were disrupted which resulted in ghosts containing only the actin cytoskeleton. Also, actin microfilaments were found to be more stable at acidic pH whereas the microtubules were found to be more stable at alkaline pH (Collings et al. 1998). Aldehydes like formaldehyde and glutaraldehyde are the most common chemical fixatives used in experiments. Formaldehyde is capable of cross-linking reaction between amino, imino, amido, guanidyl and hydroxyl groups and aromatic rings of proteins (Pearse 1980; Yamashita 2007). The effect of formaldehyde on cross-linking was demonstrated by SDS-PAGE experiments (Hopwood et al. 1988; Rait et al. 2004; Yamashita and Okada 2005). These alterations lead to changes in the three-dimensional structure of proteins, thus affecting the tertiary and quaternary structures of protein, whereas the primary and secondary structures remain unaffected (Dill and Shortle 1991; Mason and O'Leary 1991; Werner et al. 1996). The two functional groups of glutaraldehyde causes the formation of a stable and irreversible crosslinks (Eltoum et al. 2001). Glutaraldehyde affects the α helix structure of the proteins because of its strong fixing ability (Yamashita 2007). Because of these alterations, the epitopes of a protein become “masked” which makes them inaccessible to the antibodies. Essentially, this does not mean that the epitopes are critically altered. Antigen retrieval techniques could be a useful tool to significantly increase the sensitivity for detection of epitopes for better morphological detail and more precise antigen localisation (D'Amico et al. 2009; Iwamura et al. 1994; Sheriffs et al. 2001; Van den Berg et al. 1993). This property can be an important tool to achieve better visualisation of the interaction between actin microfilaments and actin-binding proteins

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